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HEMOLYTIC ACTION OF A STAPHYLOCOCCUS DUE TO A FAT-SPLITTING ENZYME.

By MARION L. ORCUTT and PAUL E. HOWE, Ph.D.

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(Received for publication, September 2, 1921.)

In the routine examination of a sample of milk two dilutions plated in blood agar showed different effects. The plate of a 1:100 dilution indicated a pure culture of a non-hemolytic staphylococcus, while the plate of a 1:10 dilution appeared to be a pure culture of a hemolytic staphylococcus. In the latter case the deep as well as the surface colonies showed clear hemolytic zones with a few corpuscles remaining next to the colony. This hemolysis is unusual for a staphylococcus which, ordinarily, even when hemolytic on the surface does not give evidence of hemolysis around the deep colonies. A culture was prepared by inoculation from a single deep hemolytic colony. The platings were repeated with this culture, and the same results obtained. These observations indicated that some constituent of the medium was responsible and not the presence of both a hemolytic and a non-hemolytic organism. The following study was, therefore, made to determine more accurately the precise factors involved.

The relation of the milk to the hemolytic process was outlined by plating the culture on (*a*) plain blood agar, (*b*) blood agar plus 0.1 cc. of sterile fat-free milk, (*c*) blood agar plus 0.1 cc. of whole milk, and (*d*) blood agar plus 0.02 cc. of cream. The results given in Table I show that hemolysis did not occur around the deep colonies in either the plain blood agar or in that to which fat-free milk had been added, but it did occur in the plates made with whole milk and with cream. Similar results occurred when bouillon was used instead of agar. They indicated that the effect of the dilution noted originally corresponded to a lowered fat content. The problem, therefore, resolved itself into a question of the relation of the fat to hemolysis and the action of the organism on the fat.

Characteristics of the Organism from Milk, Designated Staphylococcus A.—The organism is a small Gram-positive coccus, occurring often in pairs and irregular masses, and frequently in groups of three. On an agar slant the growth is smooth, opaque, and slightly yellowish white. On a blood agar plate the surface colonies are round, opaque, smooth, and more distinctly yellowish in color. In plain bouillon there is a good clouding, the growth adheres to the sides of the tube often forming a ring at the surface, and there is a small amount of sediment. Gelatin is liquefied after about 2 weeks. Milk is coagulated in 5 to 15 days. Acid is formed in glucose, lactose, sucrose, maltose, and mannitol bouillon, but no gas is produced. All these characteristics identify the organism as a staphylococcus.

TABLE I.

Hemolytic Action of Staphylococci in Media Containing Defibrinated Horse Blood with and without Cream.

	Control without milk.	Fat-free milk. 0.01 cc.	Whole milk 0.01 cc.	Cream 0.002 cc.
Blood agar plus culture (plated).....	−*	−	+	+
" bouillon plus culture.....	−	−		+++
" "	−	−		−

In the case of the agar the results are given for the deep colonies only. The quantities of milk, etc., indicated are those added to 1 cc. of media.

* In all the tables + indicates hemolysis; − no hemolysis.

Preparation of Blood.—The blood corpuscles obtained from horse blood were washed with 0.85 per cent salt solution and suspended in this fluid made up to the original volume of the defibrinated blood. It was added in the proportion of 1 drop to a cc. of bouillon. Whole defibrinated horse blood was used in the agar plates, about 1 cc. to each plate.

Standard agar was used for the plates in 10 to 12 cc. amounts. Standard bouillon was used for the bouillon cultures. 1 cc. of bouillon was measured into each tube. 0.02 cc. of 15 per cent cream diluted to 1:10 and 1 drop of blood were added to each cc. All tubes were incubated at 37°C. over night.

The results of the experiments are contained in Tables I to VI. The nature of the hemolytic zone on the blood agar plates suggested that the active hemolytic agent diffused out from the colonies; *i.e.*, that the process was extracellular since the hemolytic zone extended for a considerable distance beyond the colony. To determine the presence of an extracellular hemolytic agent the experiments summarized in Table II were carried out.

The staphylococci were killed and any extracellular enzymes present left unharmed. Such a procedure excluded the use of heat. We adopted ether, and later chloroform for control work, as an agent which would destroy the staphylococcus and which could then be removed from the media and thus would not interfere with the subsequent experiments because of its own hemolytic action. The

TABLE II.

Hemolytic Action of Bouillon Cultures of Staphylococci Sterilized with Ether or Chloroform to Which Horse Blood Corpuscles and Milk Fat Were Added.

	Cream (15 per cent) 0.002 cc.	Fat-free milk 0.01 cc.	Control without fat.
Etherized bouillon culture plus corpuscles.....	+++	−	−
Chloroform " " " "	+++	−	−

preparation of these etherized cultures was as follows: The organism was grown in standard bouillon 24 to 48 hours. Ether was added and the culture fluid shaken and allowed to stand at room temperature. The next day the sterility was tested by inoculating a tube of bouillon with 2 or 3 drops of the etherized culture. The ether was driven off by blowing air into the tube through a sterile pipette plugged with cotton. This procedure was repeated three or four times by alternately warming the culture in the incubator and then blowing air through it while cooling. This drove out practically all ether. That the specific effect of the ether was eliminated was indicated by the control tests. The results obtained with the etherized cultures, and confirmed with cultures treated with chloroform, show that after the staphylococcus has grown in bouillon the agent destroying the living organism does not destroy the substance which, in conjunction with

the cream or fat, produces hemolysis of red blood corpuscles. In the absence of cream or a fat hemolysis fails to occur.

It seemed evident that we were dealing with a lipolytic enzyme. In such a case if sufficient heat be applied either to the living culture or to the killed culture hemolysis should be prevented. The data indicating the effect of heat are summarized in Table III.

TABLE III.

Hemolytic Action of Both Living and Killed Bouillon Cultures of the Staphylococcus Which Had Been Heated before the Cream and Blood Corpuscles Were Added.

	Temperature.	Time.	Hemolytic action.
	°C.	*min.*	
Living culture	Unheated.		+++
	45	30	++*
	65	30	−
	100	5	−
Culture killed by ether	Unheated.		++
	45	30	++
	55	30	++
	65	30	−
	100	5	−
" " " chloroform	Unheated.		+++
	45	30	+++
	55	30	+++
	65	30	−
	100	5	−

Final concentration of cream was 0.002 cc. per cc. of culture fluid.
*After heating to 45°C. the culture continued to grow.

It appeared at first that hemolytic action in the etherized culture was destroyed when the fluids were heated to 45°C. Later tests showed that the first results were due to the fact that the fat added to the tube after heating rose to the surface and was, therefore, acted upon very slowly. By occasionally shaking the tubes hemolysis occurred in tubes heated at 45°C. and also at 55°C., but at 65°C. hemolytic action was destroyed.

Having found that the ultimate hemolytic process was extracellular and thermolabile the next step was to eliminate the enzyme from the

final stage of hemolysis. This was done by allowing the living staphylococcus or the etherized culture to act upon cream or fat and then to destroy the organism or enzyme with heat before adding blood corpuscles. It was found that when the living culture or the etherized culture fluid was heated to 100°C. for 5 minutes neither of the fluids thus treated would hemolyze red blood corpuscles. On the other hand, when the living culture was in contact with cream for 4 hours and the etherized culture with cream for 18 hours and these fluids were then heated to 100°C. for 5 minutes hemolysis resulted. Success with this phase of the problem was no doubt due to the absence of complex protein material in the bouillon. To demonstrate this fact small amounts of serum were added to previously heated tubes of bouillon which were hemolytically active, and the resulting mixture was heated to 100°C. The addition of 1 or 2 drops of serum to 1 cc. of bouillon had no effect upon the hemolytic action of the bouillon; the addition of 4 drops resulted in a slight hemolysis, while the addition of 7 drops of serum resulted in a failure of the heated culture fluid to hemolyze the red blood corpuscles.

The substrate for the action of the lipase in the previous experiments was cream. To eliminate cream as a factor *per se*, other fats were tested with the staphylococcus and with the etherized culture fluid, such as butter, olive oil, nut butter, triolein, triacetin, tributyrin, and pork fat. The results are contained in Table IV.

Preparation of Fats.—Preliminary experiments suggested that a high degree of emulsification of fat was desirable. Soaps of certain fatty acids will lake red blood cells, consequently emulsification with an alkali was not feasible. Fair results were obtained by shaking various fats or oils with bouillon. The quantity of fat used was such that the concentration would approximate the quantity of fat added to the culture when 15 per cent cream was used.

Cream was drawn from the top of a tube of milk after it had stood several hours. This was sterilized fractionally in an Arnold sterilizer at 100°C. for 20 minutes on 3 successive days. For use it was diluted to 1:10 with sterile bouillon, making approximately a 1.5 per cent suspension. Fat-free milk was obtained by centrifuging and lifting off the cream with a spatula. This was sterilized fractionally. A suspension of olive oil was made by adding 1.5 cc. to 10 cc. of bouillon

and shaking in a mechanical shaker 1 to 2 hours. Suspensions of other fats, such as butter, nut butter, triacetin, triolein, tributyrin, and pork fat, were made in a similar way in bouillon and shaken. The final concentration of the fats in the experimental tubes was approximately 0.002 cc. per cc. of bouillon.

Positive results were obtained with emulsions of butter, olive oil, and triolein. With some fats the hemolytic action was less marked than with others. The difference in the action seems to be due, in part at least, to the character of the emulsion, for with those fats which were associated with hemolysis the more highly emulsified they were the better the results; furthermore, a shaken tube would often give

TABLE IV.

Hemolytic Action of Living and Etherized Bouillon Cultures of the Staphylococcus to Which Were Added Horse Blood Corpuscles and Various Fats.

	Cream.	Butter.	Olive oil.	Tributyrin.	Triolein.	Triacetin.	Nut butter.	Pork fat.	Fat-free milk.	Control without fat.
Living bouillon culture plus corpuscles	+++	+	++	−	++	−	−	−	−	−
Etherized bouillon culture plus corpuscles	++	+	+		+		−	−	−	−

Final concentration of fat was approximately 0.002 cc. per cc. of bouillon.

a greater degree of hemolysis than an unshaken tube. There is considerable evidence in the literature to the effect that the salts of unsaturated fatty acids hemolyze red blood corpuscles more readily than the saturated fatty acids. Our results are, in general, in harmony with such findings.

To confirm our results with Staphylococcus A other strains indicated below were tested.

Staphylococcus B: A strain recently isolated from the milk of a cow. This cow and the one from which Staphylococcus A was isolated were in the same herd. The characteristics are in general the same as those for Staphylococcus A.

Staphylococcus C: An organism from our collection of stock cultures, isolated from a lung abscess of a calf in 1918. It liquefied

gelatin but not blood serum, and showed the yellowish color of a *Staphylococcus aureus*.

Staphylococcus D: A non-hemolytic organism also from our collection, isolated from purulent milk in 1899. It shows a yellowish pigment characteristic of a *Staphylococcus aureus*.

The experiments previously described were repeated with the different strains. The data for the bouillon culture experiments are contained in Table V.

TABLE V.

Hemolytic Action of Different Strains of Staphylococci in Bouillon Containing Defibrinated Horse Blood with and without Fat.

	Strain.	Incubation period.	Cream.	Butter.	Olive oil.	Tributyrin.	Triolein.	Triacetin.	Fat-free milk.	Control without fat.
		hrs.								
Blood bouillon plus culture..	Staphylococcus B	24	++++	+	+++	−	+++		−	−
		48	++++	+++	++++	−	++++	−	−	−
	" C	24	++	+	±	−	+	−	−	−
		48	++++	++	+	−	++	−	−	−
	" D	48	−	−	−	−	−	−	−	−
Blood bouillon only.....			−	−	−	−	−	−	−	−

Final concentration of fat was approximately 0.002 cc. per cc. of bouillon.

The hemolytic action of Strains B and C was practically identical with that given for Staphylococcus A. Hemolysis occurred to a slight extent on the surface of agar plates in the absence of added fat, and was absent around the deep colonies or at most only a faint trace of hemolysis occurred. In the presence of cream, hemolysis was more extensive on the surface and occurred around the deep colonies. In bouillon, hemolysis occurred only in the presence of fat. With bouillon cultures killed with ether or chloroform the resulting fluid was able to effect hemolysis in the presence of cream. The fluids from cultures which had acted upon cream and had then been heated to 100°C.

were capable of hemolyzing red blood corpuscles. The active agent was destroyed by heating to 65°C.

Strain C which has been under cultivation for 3 years was not as active as the more recently isolated Strains A and B. The quantity of lipase elaborated in a given time was not so great, as evidenced by its slower rate of hemolysis in the bouillon culture tubes. In the tests on different kinds of fats it was found that hemolysis occurred with olive oil only after 65 hours.

TABLE VI.

Hemolytic Action of Staphylococcus A in Media Containing Blood Corpuscles from Various Animals with and without Cream.

		Horse.	Rabbit.		Sheep.	Cow.	Calf.
			A.	B.			
Agar plus culture	Without cream.	−	−	±	−	−	−
	With "	+	+	+	+	+	+
Living bouillon culture	Without "	−	+	+	+	+	−
	With "	+	+	+	+	+	+
Etherized bouillon culture	Without "	−		+	−		−
	With "	+		+	+		+
Heated bouillon culture (100°C.)	Without "						
	With "	−		+	−		−
Control with sterile bouillon		−		−	−	−	−

Final concentration of cream was approximately 0.002 cc. per cc. of bouillon. Results with agar are given for action around deep colonies.

Strain D was non-hemolytic and failed to produce hemolysis under any conditions.

The preceding experiments had been conducted with the corpuscles of one species of animal, the horse. Other animals were bled, such as sheep, rabbit, cow, and calf, and the experiments repeated with their corpuscles. The data are contained in Table VI.

In the presence of the blood corpuscles of sheep, rabbit, cow, and calf when the organism was plated on agar without the addition of cream, only a slight hemolysis occurred around the surface colonies

and no hemolysis around the deep colonies, except in the case of rabbit blood in which a very narrow hemolytic zone was present around the deep colonies. In those plates to which cream had been added hemolysis was more extensive around the surface colonies and took place around the deep colonies in a manner very similar to that for horse corpuscles. In plain bouillon with the corpuscles of sheep, rabbit, and cow, hemolysis occurred with the living culture of Staphylococcus A in the absence of added fat, while the result with calf corpuscles was like that with the horse. When the etherized culture fluid without cream was used, hemolysis did not occur with any of the blood corpuscles except in the case of those of the rabbit; in the presence of cream, hemolysis occurred in all cases and the extent of hemolysis with the rabbit corpuscles was much greater than in the absence of cream. Rabbit corpuscles were also hemolyzed by the heated culture. In view of the greater sensitiveness of the rabbit corpuscles, and the increased hemolysis in the presence of cream or added fat, it is not illogical to assume that there was sufficient fat in the bouillon to enable the staphylococcus to effect hemolysis through the hydrolysis of this fat. The difference between the blood corpuscles of various kinds may be due to the amount or character of fat contained in the corpuscles.

In the above experiment with the corpuscles of animals other than the horse it was noted that in the absence of cream slight hemolysis occurred around the surface colonies but not around the deep colonies. In the presence of cream, hemolysis occurred around the deep as well as the surface colonies. To eliminate the possible effect of oxygen of the air as a factor in the latter process agar shake cultures were made and pipetted into flattened test-tubes filled with the agar to a height of about 4 inches. Hemolysis took place in the bottom of such tubes as well as near the surface.

DISCUSSION.

From the experiments presented hemolysis in the case of the organism studied is shown to be associated with the presence of fat in the media. Cultures grown in plain horse blood bouillon or in the presence of fat-free milk are not hemolytic, while the cultures grown in the presence on cream or other fats are hemolytic.

The nature of the processes associated with the hemolytic action is brought out in the experiments dealing with culture fluids in which the organisms have been treated with ether, and by the effect of heat upon such fluids and on living cultures. Through the action of ether or chloroform, the organism itself is killed but this does not destroy the ability of the culture fluid to effect the hemolysis of the red blood corpuscles. If the living culture or an etherized culture fluid be heated neither of the resulting fluids is capable of hemolyzing the red blood corpuscles.[1] When a living culture or an etherized culture fluid of the staphylococcus is permitted to stand with cream or other fat for several hours and is then heated to 100°C. the resulting fluid is capable of producing hemolysis. We feel justified, therefore, in attributing the hemolytic effects to the direct action of fatty acids (or soaps) which have been formed from the cream or fat by an extracellular enzyme elaborated by the staphylococcus. The enzyme is not dialyzable, for hemolytic action could not be obtained with either living or killed cultures in collodion bags.

The occurrence of hemolysis due to the action of a lipase upon fat necessitates the consideration of two factors in the study of organisms, (*a*) the presence of a lipase and (*b*) the elimination of its lipolytic action before concluding that hemolysis is due to another type of hemolytic agent.

Many points brought out by our experiments have been observed separately by others but so far as we can find the hemolytic action of a staphylococcus has not been directly connected with its lipolytic activities. Several organisms were reported by Eijkman (1) in 1901 as producing a lipase and among these was given *Staphylococcus pyogenes aureus*. An association between hemolysis and lipolysis was demonstrated by Noguchi (2) in 1907 who showed that hemolysis may be a direct result of fat-splitting. He used various tissue lipases with a considerable number of fats, but the most satisfactory results were obtained with triolein, butter, and fatty mixtures which he extracted from the fat tissues and mesentery of dogs and guinea pigs. According to his report neither the fat nor the lipase alone produced hemolysis.

Von Liebermann (3) extracted a thermostable hemolytic substance from hog corpuscles which was found to be an acid or a mixture of acids. He did not identify the acids but was able to demonstrate similar hemolytic action with

[1] There was one exception to this statement and that was the corpuscles of the rabbit (see page 417).

oleic acid. Heating inactivated oleic acid-serum mixtures. This agrees with the work of Noguchi who found that when his alcohol extracts (soap) were heated alone they were not inactivated but when heated with serum they were inactivated. Von Liebermann was able to reactivate such mixtures by the careful addition of acid.

Neuberg and Reicher (4) demonstrated the lipolytic action of various plant and animal substances by measuring the increase in acidity of mixtures of an hemolysin and a fat, particularly olive oil. These authors suggest that the hemolytic effects observed by von Liebermann may have been due to the fatty acid produced by the action of lipase upon the fats of red blood corpuscles. In our experiments the hemolytic effect is apparently not directly upon the red blood corpuscles, since in our control experiments hemolysis failed to occur in the presence of lipases but in the absence of added fat.

Noguchi (5) also extracted a hemolytic agent from animal tissues with alcohol which consisted of soaps. Fatty acids or their soaps are known to produce hemolysis. The smallest amount of fatty acid necessary for complete hemolysis of 0.5 cc. of a 5 per cent suspension of red blood cells was studied by McPhedran (6) in 1913. In our experiments we made no quantitative determinations but we used about 0.3 mg. of the fat, and probably this was not all split into fatty acids. McPhedran found 0.03 mg. of oleic, linoleic, dibromostearic, or the two isomeric monobromostearic acids sufficient for complete hemolysis of 0.5 cc. of a 5 per cent suspension of red blood corpuscles while palmitic acid and dihydroxystearic acids were required to be present in quantities ten times as great as for the above acids.

The hemolytic character of the staphylococcus is described in various text-books as due to a specific hemolysin, staphylolysin, but no mention is made of a fat-splitting enzyme.

SUMMARY.

A staphylococcus was isolated from milk which is hemolytic with horse blood only in the presence of fat. Similar results were obtained with two other strains of staphylococci.

The hemolysis is the result of the action of a fatty acid (or soap) upon the red blood cells. The fatty acid is formed by the action of a lipase elaborated by the staphylococcus.

The corpuscles of different animals show slight variations in the ease with which they are hemolyzed by the staphylococcus.

Attention is called to the desirability of testing for lipases in the study of staphylococci or of hemolysis.

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EXPERIMENTAL RICKETS IN RATS.

IV. The Effect of Varying the Inorganic Constituents of a Rickets-Producing Diet.*

By A. M. PAPPENHEIMER, M.D., G. F. McCANN, M.D., and T. F. ZUCKER.

With the Collaboration of M. Stanley-Brown, M. Strauss, B. Stimson, and E. Klein.

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Plates 21 to 24.

(Received for publication, October 31, 1921.)

In the first paper of this series it was shown by Sherman and Pappenheimer (1, 2) that rickets could be produced in rats by a diet composed of patent flour, calcium lactate, sodium chloride, and ferric citrate, and that it could be prevented by the substitution of 0.4 per cent basic potassium phosphate for an equal percentage of calcium lactate in the diet. These experiments have been continued, the diet being modified in various ways, and the present paper will report upon the bone changes which were produced by varying the inorganic constituents.

In the meantime, there has appeared a series of studies on experimental rickets from the Johns Hopkins Laboratories in which Shipley, Park, McCollum, and Simmonds (3–7) give diets which lead more or less regularly to the development of rickets in rats, and in which they also emphasize the protective rôle of the phosphate ion. A more detailed comparison of their findings will be given later.

EXPERIMENTAL.

Because of the frequency with which rats kept under ordinary laboratory conditions develop what has previously been termed spontaneous

* A preliminary report covering some phases of this work was presented before the Society of Experimental Biology and Medicine (Pappenheimer, A. M., McCann, G. F., Zucker, T. F., and Hess, A. F., *Proc. Soc. Exp. Biol. and Med.*, 1920–21, xviii, 267).

rickets, it was realized from the outset that factors other than dietary ones which might influence the experiments should be controlled as rigidly as possible. Care was taken to obtain healthy stock, from which individual rats maintained for varying lengths of time on a complete diet showed normal bones upon histological examination. Whenever possible, controls were taken from the same litters as those used for the experiment.

The animals were kept in cylindrical wire cages, 8 inches high and 9 inches in diameter, in a dimly lighted, screened room, with northern exposure.

Since these experiments were begun it has been shown by Shipley, Park, Powers, McCollum, and Simmonds (8), and by Hess, Unger, and Pappenheimer (9) that exposure to direct sunlight gives complete protection in phosphorus-low diets. Since, fortunately, the light conditions in our experiments have been kept fairly uniform, and the rats at all times excluded from intense light, this factor has been without influence upon the results. It must be strongly emphasized, however, that our data, so far as their quantitative value is concerned, hold only for rats maintained under the described conditions.

Not more than three rats were kept in the same cage. The diets were mixed in bulk, care being taken to ensure a uniform distribution of the components. It was found impracticable to weigh back the uneaten food as a routine procedure, but the amount actually consumed was roughly determined in a number of instances and found to vary very little for animals of approximately the same age and weight. It is believed that no serious errors have been introduced by this omission, since almost without exception, the rats maintained on the same diets have shown, on final examination, identical or comparable findings in the bones.

The rats were usually placed on the experimental diet at the age of 4 weeks, or about 1 week after weaning. After 4 weeks on the test diet, a radiograph was taken; and when subsequent modifications of the diet were introduced, the accompanying changes were followed radiographically. Because of the known protective effect of light, the possibility that repeated exposure to x-rays might have a curative influence had to be considered. Three rats were exposed daily over a period of 3 to 9 days, the same dosage being used that was employed in

making radiograms. The rickets, which had developed on Diet 84, was not influenced, no healing being evident on radiographic examination.

A complete autopsy was performed on each rat, the teeth, ribs, femora, tibiæ, eyes, and such viscera as showed gross lesions being preserved for histological study. Possible alterations in the teeth will be made the subject of a later report. The study of the long bones also has not been completed, but previous experience has shown that the lesions in the ribs in general parallel those found in other bones, and may safely be taken as indices of the nature and extent of the bone changes. Fig. 1 shows the microscopic appearance of the normal rib.

The difficulties in the histological examination of bones, as regards the precise differentiation of the calcified and uncalcified tissue, are too familiar to require emphasis. The routine adopted for these studies has been the following. Two lower ribs from each side were fixed for 24 hours in Müller-formol, decalcified for 5 to 10 days in Müller's fluid, and embedded in paraffin. Sections were stained with hematoxylin-eosin, and by von Kossa's silver nitrate method, applied as follows:

1.5 per cent silver nitrate—30 minutes or more in bright light.
Repeated washing in distilled water.
Counterstaining with hematoxylin-Van Gieson.

The calcium (phosphate) appears in these preparations as a dense black, the cartilage bluish purple, the osteoid tissue bright red.

The influence of the time element in the decalcification with Müller's fluid upon the stainable calcium was studied by removing ribs after varying periods from the decalcifying fluid, staining the sections identically, and comparing the results. It was found that within the range of 5 to 10 days no significant differences in the distribution of the stainable calcium could be detected.

Rickets-Producing Diet.

The rickets-producing diet, No. 84, which has served as a basis for this work, has the percentage composition indicated in Table I. The mineral composition is shown in Table II.

The original observation that rats maintained on this diet invariably develop rachitic lesions, has been tested in 55 additional animals, all

TABLE I.

Composition of the Diets Used.

Diet No.	Composition of diet.	Total No. of rats examined.	
		Rachitic.	Non-rachitic.
83	Patent flour.....95.0 per cent. Calcium lactate..... 3.0 " " Sodium chloride..... 2.0 " "	2	0
84	Patent flour.....95.0 " " Calcium lactate..... 2.9 " " Sodium chloride..... 2.0 " " Ferric citrate..... 0.1 " "	68	0
85	Patent flour.....95.0 " " Calcium lactate..... 2.5 " " Sodium chloride..... 2.0 " " *Potassium phosphate* (basic). 0.4 " " Ferric citrate..... 0.1 " "	0	46*

* Five of these were placed on special diet at the age of 60 days, one at 81 days; all remaining animals started about 1 week after weaning.

of which developed rickets as shown by x-rays, gross examinations, and by microscopic study of the ribs. (In two rats, no sections could be made.)

The preventive action of the potassium phosphate (Diet 85, Table I) has also been confirmed on thirty-one additional rats, giving a total of forty-six rats in which protection has been uniformly obtained. The mineral analysis of this diet is also shown in Table II.

Determination of the Rôle Played by Potassium and Phosphate Ions Respectively in the Protection Given by Basic Potassium Phosphate.

In this experiment, secondary sodium phosphate in the proportion of 0.8 per cent of the diet was substituted for the secondary potassium phosphate in Diet 85, this amount being calculated to contain an equivalent amount of phosphorus (0.072 gm.). Of the four rats (Nos. 88 to 91) on this diet, none developed rickets (Figs. 2 and 3). Two rats (Nos. 86 and 87) from the same litter, on Diet 84, developed marked rickets (Table III).

TABLE II.

*Calculated Mineral Composition of Diets 84 and 85.**

Mineral constituent.	Diet 84.		Diet 85.	
	Amount per 100 gm. of food.	Per cent.†	Amount per 100 gm. of food.	Per cent.†
	gm.		*gm.*	
K	0.110	3.8	0.290	9.4
Na	0.847	29.3	0.847	27.6
Ca	0.550	19.1	0.477	15.5
Mg	0.017	0.6	0.017	0.5
P	0.087	3.0	0.159	5.2
Cl	1.280	44.4	1.280	41.7
Total	2.891	100.2	3.070	99.9

* Data for white flour taken from Sherman, H. C., Chemistry of food and nutrition, New York, 2nd edition, 1918.

† On the basis that K + Na + Ca + Mg + P + Cl = 100 per cent.

Determinations of phosphorus and calcium (McCrudden's method (McCrudden, F. H., *J. Biol. Chem.*, 1911-12, x, 187) for calcium and titration of ammonium phosphomolybdate for phosphorus) on the diets as used gave the following figures.

	Diet 84. *gm.*	Diet 85. *gm.*
P	0.0868	0.1582
	0.0880	0.1593
Ca	0.5530	0.4805
	0.5521	0.4786

For another group of rats (Nos. 97 to 99), 0.35 per cent of potassium chloride was used, thus adding an equivalent amount of potassium (0.18 gm. per gm.) to the diet in the form of chloride instead of phosphate. The three rats on this diet developed rickets of the same degree of severity as did two control rats of the same litter (Nos. 100 and 101) on Diet 84 (Figs. 4 and 5).

The obvious conclusion from these results is that the protective effect of the basic potassium phosphate is attributable to the phosphate ion and not to the potassium.

TABLE III.

Effect of the Substitution of Sodium for Potassium, and of Chloride for Phosphate, in Diet 84.

Rat No.	Litter No.	Diet.	Length of time on diet.	Weight.			Radiograph.		Pathological examination.		
				Initial.	Maximum.	Final.	Day of diet.	Rickets.	Rickets.	Ophthalmia.	Other lesions.
		No. 85A.	*days*	*gm.*	*gm.*	*gm.*					
88	14	Flour.....95.0 per cent. Calcium lactate.... 2.5 " " Sodium chloride.... 1.6 " " Ferric citrate..... 0.1 " " *Sodium phosphate* (basic)..... 0.8 " "	21	31	40	40			None.*	None.*	None.
89	14		37	30	46	46	35th	None.	" *	Slight keratitis* (left).	"
90	14		37	38	47	44	25th 35th	" "	" *	Moderate.	"
91	14		37	30	47	42	25th 35th	" "	" *	Keratitis.* None.	Abscess in subinguinal gland.
		No. 85B.									
97	16	Flour.....95.0 per cent. Calcium lactate.... 2.5 " " Sodium chloride.... 2.05 " " Ferric citrate..... 0.1 " "	36	38	43	40	25th	Moderate.	Moderate.*	Slight keratitis.*	None.
98	16	*Potassium chloride*.. 0.35 " "	21	38	46	46			Slight.*	None.	"
99	16		36	32	44	40	25th	Moderate.	Marked.*	Marked keratitis.*	"

Controls from the same litters.											
		No. 84.									
86	14	Flour.....95.0 per cent. Calcium lactate. ... 2.9 " " Sodium chloride.... 2.0 " " Ferric citrate. ..... 0.1 " "	27	32	40	40	24th	Marked.	Moderate.*	Marked keratitis.*	None.
87	14		44	33	43	40	38th	"	Marked.*	Suppuration.	Suppurative rhinitis; atelectasis of lungs.
100	16		20	34	37	32			Early.*	Slight keratitis.	None.
101	16		34	30	35	35			Slight.	Conjunctivitis.	?

* Confirmed by microscopic examination.

TABLE IV.

Effect of Variations in the Amount of Phosphate Added.

Rat No.	Litter No.	Diet.	Length of time on diet.	Weight.			Radiograph.		Pathological examination.		
				Initial.	Maximum.	Final.	Day of diet.	Rickets.	Rickets.	Ophthalmia.	Other lesions.
		No. 85N.	*days*	*gm.*	*gm.*	*gm.*					
112	18	Flour.....95.0 per cent. Calcium lactate..... 2.64 " " Sodium chloride..... 2.0 " "	27	47	65	65	20th	None.	None.*	Slight corneal opacity.	None.
117	19	Ferric citrate..... 0.1 " " Potassium phosphate (basic; equal to 50 mg. per 100 gm. of diet)..... 0.26 " "	34	43	50	50	20th	"	Slight, early.*	Slight corneal opacity.	"
		No. 85O.									
114	18	Flour.....95.0 per cent. Calcium lactate..... 2.77 " " Sodium chloride..... 2.0 " "	27	42	56	53	20th	Slight.	Moderate.*	Slight corneal opacity.	None.
119	19	Ferric citrate..... 0.1 " " Potassium phosphate (basic; equal to 25 mg. per 100 gm. of diet)..... 0.13 " "	34	28	35	35	20th	"	Marked.*	Slight conjunctivitis.	"
120	?		40	34	46	46	20th	"	" *	Marked conjunctivitis; corneal opacity.	"

CORRECTIONS.

On page 429, Vol. xxxv, No. 4, under Diet 85, for *72 mg. of phosphate* read *72 mg. of phosphorus*.

		No. 85P.										
113	16	Flour.....	95.0 per cent	34	46	54	53	20th	Slight.	Marked.*	Marked conjunctivitis.	None.
		Calcium lactate.....	2.85 " "									
		Sodium chloride.....	2.00 " "									
118	19	Ferric citrate.....	0.10 " "	27	52	58	56	20th	"	Slight.*	None.	"
		Potassium phosphate (basic; equal to 10 mg. per 100 gm. of diet).....	0.05 " "									
		Controls from the same litters.										
		No. 84.										
110	18	(No phosphate added.)		27	35	40	35	21st	Slight.	Moderate.*	None.	None.
115	19			27	36	41	41	27th	Moderate.	Cod liver oil.		
		No. 85.										
111	18	(72 mg. of phosphate per 100 gm. of diet added.)		37	46	63	63	21st	None.	Transferred to Diet 84.		
								26th	"			
116	19			27	39	49	49	20th	"	None.*	None.	None.

* Confirmed by microscopic examination.

TABLE V.

Effect of the Addition of Salt Mixture Complete except for Phosphorus.

Rat No.	Litter No.	Diet.	Length of time on diet.	Weight.			Radiograph.		Pathological examination.		
				Initial.	Maximum.	Final.	Day of diet.	Rickets.	Rickets.	Ophthalmia.	Other lesions.
			days	*gm.*	*gm.*	*gm.*					
168	29	No. 84 with Osborne and Mendel salts (without H_3PO_4).	26	31	31	27	26th	Marked.	Marked.*	None.	None.
169	29	Flour.....95.0 per cent Osborne and Mendel salt mixture..... 5.0 " "	26	31	31	26	26th	"	†		
227	36		28	40	48	48			Marked.*	Slight corneal opacity.	None.
228	36		37	41	46	46	35th	Marked.	Moderate.*	Slight corneal opacity.	"
229	36		37	37	43	43	35th	"	" *	None.	"

*Confirmed by microscopic examination.

†See Pappenheimer, A. M., McCann, G. F., and Zucker, T. F., *J. Exp. Med.* 1922, xxxv, 461, Table VI.

Determination of the Amount of Phosphate Necessary for Protection.

Eleven rats (Nos. 110 to 120), including four controls, were placed on diets in which graded amounts of basic potassium phosphate had been substituted for equivalent amounts of calcium lactate in Diet 84. Additions of 10, 25, and 50 mg. of phosphorus per 100 gm. of diet in the form of K_2HPO_4 were made to the standard basic diet in this way, giving totals of 97, 112, and 137 mg. of phosphorus per 100 gm. in these diets. As shown in Table IV, of those receiving 50 mg. of added phosphorus (Rats 112 and 117), one (No. 112) showed no evidence of rickets. The other (No. 117) presents an appearance which is interpreted as slight or early rickets (Fig. 6).

The zone of proliferative cartilage is widened to six to eight cells (normally four to five). Alignment is slightly irregular and calcification of the matrix is defective in the proximal portions of the cartilage. The primary spongiosa is delicate and the trabeculæ are surrounded by narrow but distinct osteoid borders about which the osteoblasts are very conspicuous. Along the shaft, endosteal bone formation is active. Around the perforating vessels, there is a distinct osteoid border which is wider than that found in normal ribs.

The rats receiving only 10 and 25 mg. of added phosphorus per 100 gm. of diet show definite rachitic lesions (Fig. 7). The proliferating cartilage is extremely wide and irregular, but the matrix, in the distal portions, especially, is not wholly free from calcium. The trabeculæ of the spongiosa show an osteoid border greatly in excess of the normal, but the distinction between the fully calcified bone and the osteoid tissue is in many cases less sharp than in rats which have been on Diet 84. It would seem, therefore, that the small amount of additional phosphate favors the deposition of calcium to a limited extent, and that with the addition of 50 mg. per 100 gm. the balance is so delicate that individual differences come into play.

Effect of the Modification of Inorganic Constituents Other than Calcium and Phosphorus.

Rats 168, 169, 227, 228, and 229 (Table V) were given a diet composed of flour and 5 per cent of salt mixture made similarly to that described by Osborne and Mendel (10) except that no phosphoric acid was included (Fig. 8). These rats were kept on the diet for 26, 26, 28, 37, and 37 days and showed marked rickets.

TABLE VI.

Effect of the Reduction of Sodium Chloride in Diet 84.

Rat No.	Litter No.	Diet.	Length of time on diet.	Weight.			Radiograph.		Pathological examination.		
				Initial.	Maximum.	Final.	Day of diet.	Rickets.	Rickets.	Ophthalmia.	Other lesions.
		No. 84L.	*days*	*gm.*	*gm.*	*gm.*					
268	42	Flour.....96.5 per cent.	35	33	42	42	30th		Marked.*	None.*	None.
269	42	Calcium lactate. ..... 2.9 " "	35	32	40	38	30th	Marked.	" *	Very slight.	"
		Sodium chloride. ..... 0.5 " "									
		Ferric citrate. ..... 0.1 " "									
270	42		35	38	48	47	30th	"	" *	None.	"

This salt mixture as given contains an amount of calcium which is approximately that used in Diet 84, and it also contains all the other inorganic elements considered necessary for adequate growth, with the single exception of phosphorus. The development of rickets in the rats would seem to indicate that these other elements have no protective action.

The basic rickets-producing diet, No. 84, contains 2 per cent of NaCl, an amount probably considerably in excess of the requirement. It was conceivable that this excess might in some way be a contributory factor in the production of the rachitic lesions, and to settle this point, three rats (Nos. 268 to 270) were given Diet 84L, in which the NaCl was reduced from 2 per cent to 0.5 per cent. After 30 days all three rats had developed typical rickets, as revealed by gross and microscopic examination. A reduction of the NaCl in Diet 84 to 0.5 per cent, therefore, did not prevent the development of active rickets (Table VI).

Effect of Variations in the Proportion of Calcium and Phosphorus in Diets 84 and 85.

Although it had become evident from the preceding work that the presence or absence of rachitic lesions could be determined by varying the phosphate ion in the diet, when the calcium was present in excess, and that the other inorganic constituents of the food were without apparent influence, it seemed of importance to ascertain what modifications in the structure of the bones might be brought about by varying the relative proportion of calcium and phosphate in the diet.

The literature, it is true, contains many references to the structural changes produced in the bones by a calcium-deficient diet, but in general, the phosphate content of the diet in relation to the calcium has not been considered. Furthermore, aside from the recent papers of McCollum and his associates, no data referring to the effects of deficiency of one or both of these inorganic constituents upon the bone structure of rats were available.

A summary of our experiments is given in Table VII. In the first series (Rats 233 to 235), the calcium lactate and basic potassium phosphate of Diet 85 were reduced, the former to 1.4 per cent (209 mg. per 100 gm. of diet), the latter to 0.26 per cent (49 mg. of added P, or a total of 136 mg. per 100 gm.).

TABLE VII.

Effect of Variations in the Proportion of Calcium and Phosphorus in Diets 84 and 85.

Rat No.	Litter No.	Diet.	Length of time on diet.	Weight.			Radiograph.		Pathological examination.		
				Initial.	Maximum.	Final.	Day of diet.	Rickets.	Rickets.	Ophthalmia.	Other lesions.
		No. 85 + 209 mg. of Ca.	*days*	*gm.*	*gm.*	*gm.*					
233	38	Flour.....96.5 per cent.	41	31	54	54	28th	Slight.	Slight, healing.*	None.*	None.
234	38	Sodium chloride..... 2.0 " " *Calcium lactate*..... 1.14 " "	41	35	68	67	28th	Moderate.	Healing.*	"	"
235	38	*Potassium phosphate* (basic)..... 0.26 " " Ferric citrate..... 0.1 " "	30	31	46	46	28th	"	Slight, healing.*	" *	*Hymenolepis murina.*
		No. 85 + 149 mg. of Ca.									
239	38	Flour.....96.97 per cent. Sodium chloride..... 2.0 " " *Calcium lactate*..... 0.8 " " *Potassium phosphate* (basic)..... 0.13 " "	30	30	53	53	28th	Moderate.	None* (increase in width of preparatory cartilage).	None.	None.
240	38	Ferric citrate..... 0.1 " "	42	28	50	50	28th	Slight.	None* (increase in width of preparatory cartilage).	" *	Suppurative nephritis.
		No. 84 + 111 mg. of Ca.									
236	38	Flour.....97.3 per cent.	30	34	52	52	27th	Marked.	Marked.*	None.*	None.
237	38	Sodium chloride..... 2.0 " " *Calcium lactate*..... 0.6 " "	42	29	58	55	27th	"	" *	"	"
238	38	Ferric citrate..... 0.1 " "	42	27	54	54	27th	"	" *	"	"

* Confirmed by microscopic examination.

At the end of 4 weeks, the radiographs showed that all three rats had developed an active rickets. Rat 235, killed at this time, had a mild healing rickets, with calcium deposition in the cartilage and in the osteoid surrounding the trabeculæ of the spongiosa. Rat 234, allowed to live for 41 days, showed microscopically definite healing, the cartilage and the greater portion of the osteoid being completely calcified. Rat 233, killed on the 42nd day, likewise showed healing rickets, the calcification of the cartilage and osteoid being somewhat less advanced than in the ribs of Rat 234.

In this experiment, the total P of the diet (136 mg.) was practically at the border-line content which previous experiment (Table IV, Rats 112 and 117) had shown to confer protection in some rats and not in others. The results, therefore, are not surprising—rickets after 4 weeks, with adequate healing after 41 days. It does not appear that the coincident reduction of the calcium has materially modified the lesions.

In two other rats of the same litter (Nos. 239 and 240) the calcium lactate of Diet 85 was reduced to 0.8 per cent (149 mg. of Ca per 100 gm.), and the basic potassium phosphate to 0.13 per cent (total P of 111 mg. per 100 gm.).

Again, both rats showed by radiograph, on the 28th day, an appearance interpreted as moderate rickets. Rat 239, killed on the 30th day, proved not to have rickets, the cartilage, spongiosa, and cortex being normally calcified. There was, however, an increase in the depth of the zone of preparatory cartilage to ten or twelve cells, which explains the defect noted in the radiograph. Rat 240, killed on the 42nd day, was found to have practically complete healing of the rachitic lesions in the ribs.

The zone of preparatory calcification, though wider than normally, averaging twelve to sixteen cells, and continued into several irregular prolongations, showed a complete calcification of the matrix. The primary spongiosa was somewhat defective, the secondary spongiosa better developed. In both, calcification was complete, there being no osteoid tissue about the trabeculæ. The cortex also showed no osteoid border. The trabeculæ in the metaphyseal area were separated by a very abundant, loose fibrous marrow. The perichondral osteoid was still calcium-free.

The conditions found in this rat may instructively be compared with those in Rat 120, maintained for 40 days on Diet 85O, containing an equal amount of P, but almost four times as much Ca. So far as it is permissible to draw inferences from a single experiment, it would seem that the healing of the rachitic lesions was favored by the reduction of the calcium; or otherwise stated, that the excess of calcium in the diet tended to maintain the rachitic condition.

In another series (Rats 236 to 238), no additional phosphate was given, but the calcium lactate of Diet 84 was reduced to 0.6 per cent (111 mg. of Ca per 100 gm.). The three rats, after 30, 42, and 42 days respectively, developed rachitic lesions typical histologically, and not differing from those observed on the unmodified Diet 84. The deduction from this experiment is that the Ca may be reduced to approximately one-fifth that of Diet 84, and if there is a concomitant P deficiency, the resultant lesions will be rachitic and not osteoporotic.

Effect of a Deficiency of Calcium with an Excess of Phosphate.

The material for this experiment consisted of fourteen rats, examined after periods of 29 to 36 days, upon the following diet.

Diet 85C.

Patent flour	95.0 per cent.
Sodium chloride	2.0 " "
Potassium phosphate (basic)	2.9 " "
Ferric citrate	0.1 " "

This diet led to alterations in the structure of the ribs resembling the usual rachitic changes on Diet 84, but with certain striking and interesting differences (Fig. 9). The zone of preparatory calcification of the cartilage in many of the rats was but slightly greater than in normal ribs, and in some cases within the normal range, not exceeding five or six cells. In only two rats of this series (Nos. 243 and 244) did the increased depth approximate that seen on Diet 84.

The calcium deposition in the matrix of the cartilage was also decidedly more abundant than in rats on Diet 84. In some, the calcium was found throughout the zone of preparatory calcification, in others it was limited to the matrix between the distal cells. In general, there was a much more adequate preparatory calcification of the cartilage

than was obtained in rats upon a diet rich in calcium, but deficient in the phosphate ion.

The alterations in the bony portions of the ribs, on the other hand, did not differ materially from those occurring in rats on Diet 84. There was an excessive amount of calcium-free osteoid tissue produced, not only about the trabeculæ in the subchondral region, but along the shaft as well. The osteoblasts were numerous and conspicuous.

The following tentative explanation is presented. It is probable that the matrix of the cartilage in the zone of provisional calcification has a greater avidity for both the calcium and phosphate ion than the osteoid tissue of the bone itself, since it has been observed that in the process of healing under the influence of cod liver oil, or following the addition of phosphate to Diet 84, the deposition of calcium in the cartilage precedes that in the osteoid tissue. This partial calcification of the cartilage suffices to guide the blood vessels towards a more orderly destruction of the cartilage columns, and to prevent the unrestricted and highly irregular growth of the cartilage towards the diaphysis, which takes place when, as in Diet 84, the cartilage matrix remains permanently free of calcium.

To sum up this experiment, then, we may conclude that a diet containing an abundance of phosphate, but deficient in calcium, leads to changes in the bones which resemble those of rickets, but differ from them in the fact that the endochondral changes are relatively slight or absent.

The typical findings on this diet are illustrated by the following protocol.

Rat 245.—36 days on Diet 85C. Initial weight 35 gm. Final weight 38 gm. The radiograph, taken on the 36th day, showed a narrow defect in the upper epiphyses of the tibiæ.

Macroscopic Examination.—Nutrition poor. *Eyes.*—Normal. *Viscera.*—Normal. *Ribs.*—Slight chondrocostal swelling, without angulation. The epiphyseal line is quite sharp. There are several fusiform callus beads. The bones are soft. *Tibia.*—The upper extremity is moderately swollen, and there is an increased amount of soft spongiosa in the subchondral region.

Microscopic Examination.—Rib.—The preparatory cartilage is very little widened, not exceeding eight cells (normally two to six). The columnar arrangement of the cells is maintained. Calcium deposition is limited to the matrix between the distal two or three cells. The subchondral zone is occupied by broad

masses of osteoid, the marrow in this region being reduced to a few vascular clefts. The osteoblasts are conspicuous and, in many places, surrounded by osteoid. The centers of the osteoid trabeculæ are composed of calcified bone and cartilage. The junction between calcified bone and osteoid is fairly sharp, although there is often a granular purple haze surrounding the calcified bone which suggests that the adjacent osteoid is not wholly free from calcium. The cortex is much thinned in places and even completely interrupted. It is everywhere surrounded by a wide osteoid margin. *Tibia.*—Upper epiphysis: The lesions here are very slight. The zone of preparatory calcification of the cartilage is little, if at all, increased in depth, not exceeding six cells, and the cartilage cells show a very orderly columnar alignment. There are no projections, the line being even and sharp. Calcification of the matrix is complete in the distal three or four cells. The spongiosa is quite dense. There are numerous small trabeculæ, well ossified, but with slightly increased osteoid borders. The osteoblasts are swollen and very distinct; there appears to be very active osteogenesis. Cortex further along the shaft is well calcified. There is active endosteal bone formation, with a distinct, but hardly excessive osteoid margin. The rachitic changes are insignificant as compared with those in the ribs.

Diagnosis.—Atypical rickets.

Effect of a Deficiency of Both Calcium and Phosphate.

The following diet was used for this study.

Diet Q.

Patent flour	97.9 per cent.
Sodium chloride	2.0 " "
Ferric citrate	0.1 " "

The estimated Ca content is 0.018 gm., as compared with 0.553 gm. of Ca content in Diet 84. The P content is approximately 0.088 gm. per 100 gm., or about five times that of the Ca. Eleven rats (Table VIII) were placed on this diet for periods varying from 20 to 52 days.

In our preliminary paper (1), it was stated that rats upon this, or rather a similar diet, without the addition of the ferric citrate, developed an osteoporotic condition of the bones, and not a true rickets. Further experience leads us to modify this statement somewhat. Of the nine additional rats, one only (No. 254) showed lesions which may be regarded as purely osteoporotic.

The conditions in the remaining rats differ in certain features from those of simple osteoporosis, and from rickets, as seen in rats on Diet 84. In the radiographs, the defect at the upper extremity of the tibia

TABLE VIII.

Effect of a Deficiency of Both Calcium and Phosphorus.

Rat No.	Litter No.	Diet.	Length of time on diet.	Weight.			Radiograph.		Pathological examination.	Remarks.
				Initial.	Maximum.	Final.	Day of diet.	Rickets.	Rickets.	
			days	*gm.*	*gm.*	*gm.*				
796	?	Patent flour. . . . . 98.0 per cent. Sodium chloride. . . . . 2.0 " "	52	41	44	44			Atypical.*	
799	?		52	35	39	35			" *	
		Diet Q.								
252	40	Patent flour. . . . . 97.9 per cent. Sodium chloride. . . . . 2.0 " " Ferric citrate. . . . . 0.1 " "	34	39	51	?	31st	Slight defect.	Atypical.*	Ate part of rat.
254	40		21	37	39	33			Simple osteoporosis.*	
255	40		34	31	38	38	31st	Slight defect.	Slight, atypical.*	Ate part of Rat 254.
480	61		26	52	60	59	26th	" "	Atypical.*	
481	61		32	60	71	71	26th	" "	" *	
482	61		32	57	73	73	26th	" "	" *	
492	62		32	27	38	38	26th	" "	" *	
493	62		26	26	37	37	26th	" "	" *	
494	62		32	27	44	44	26th	" "	" *	
483	61	Full.	35	58	138	138	30th	None.	None. *	
484	61		35	56	134	134	30th	"	" *	
490	61	No. 84.	26	50	57	57			Typical*	

* Confirmed by microscopic examination.

was very much narrower than in the typically rachitic rats. The swelling of the chondrocostal junctions was slight or absent, and there was no angulation or other deformity. Infractions with callus beads were seen in some of the rats, and were absent in others. The bones were delicate and fragile, rather than pliable.

Histologically, the zone of preparatory calcification was somewhat increased in depth, but not to the same extreme degree as with Diet 84, averaging only eight to twelve cells, whereas in the typically rachitic rats on Diet 84 it frequently was more than 50 cells deep. Calcification of the matrix was either absent entirely or limited to the distal portion of this zone and to the short prolongations into the metaphysis. The perichondral osteoid was only moderately increased. The trabeculæ of the subchondral spongiosa showed a wide osteoid margin, and there was a moderate increase in the cortical osteoid. Evidences of increased osteoclastic resorption were not found. The marrow showed no unusual features (Fig. 10).

These findings in general resemble rather closely those with Diet 85C, in which a deficiency of calcium is combined with an excess of phosphate. It should be noted that even in Diet Q, the proportion of total phosphorus to calcium is still in excess of that in $Ca_3(PO_4)_2$.

Effect of a Deficiency of Phosphate upon the Bones of Adult Rats.

Although somewhat apart from the main problem, it may be of interest to describe briefly the changes in the bones of mature rats after the cessation of epiphyseal growth, when placed upon a phosphorus-deficient diet. Three such rats (Nos. 22, 55, and 56) have been studied. Rat 22, after 182 days on a complete diet, during which period it had shown normal growth, was placed for 10 days on Diet 84. Rats 55 and 56, after 126 days on a complete diet, were placed for 42 days on Diet 84.

The histological changes in the ribs of these three rats were practically identical. The zone of preparatory calcification was very narrow (three to four cells), and the cells were separated by a densely calcified matrix. The abnormality noted was a definitely increased osteoid margin about the trabeculæ of the spongiosa and along the endosteal surface of the cortex. This osteoid tissue, which was very sharply differentiated from the calcified bone, was bordered externally by distinct

osteoblasts. Without entering into the still debatable question as to whether this calcium-free osteoid is to be regarded as newly formed, or whether it results from a decalcification of the already calcified bone, we may record simply that phosphorus deficiency manifests itself in the adult rat by an increase in the amount of osteoid, and that the calcification of the cartilage remains for at least 42 days unimpaired. It is well recognized that endosteal bone formation persists, though at a more sluggish rate, throughout adult life; and the simplest explanation of the appearance described is that the newly formed bone, in the absence of an adequate supply of phosphate, remains uncalcified.

DISCUSSION.

The main facts brought out in this study may now be briefly reviewed. The basic observation has been amply confirmed that lesions of the bones identical with those previously described in rats as spontaneous rickets, may be unfailingly produced by subjecting the animals to a diet containing an adequate amount of calcium, but deficient in phosphates. When the phosphate ion is added in adequate amount (equivalent to 75 mg. of P per 100 gm. of diet), the development of rickets is invariably prevented. The border-line, where individual differences come into play, is in the neighborhood of 50 mg. of added P, and when less than this is given, rachitic changes in the bones result.

It could easily be shown, moreover, that neither the potassium in itself, nor any of the other inorganic salts which enter into a complete diet, confer any protection, if there is at the same time a deficiency of the phosphate ion.

Equally definite and striking, though possibly bearing less upon the problem of human rickets, has been the observation that lesions resembling rickets may also follow the administration of a diet deficient in calcium, but containing an adequate or excessive amount of phosphate. The lesions observed have differed from those resulting from a deficiency of phosphate in the presence of an adequate or excessive amount of calcium, in that the endochondral changes are less marked. The lesions which resulted from a diet highly deficient in both calcium and phosphorus differed from the more typical rickets in degree rather than in kind. With one exception, there developed a moderate increase in the depth of the zone of preparatory calcification, with defective

deposition of calcium, and an excess of osteoid in the spongiosa and cortex. There was no clear anatomical evidence of an increased resorption of calcium. The effects of altering the proportion of calcium and phosphate in the diet are, perhaps, less easily interpreted. It seems certain that a marked excess of Ca, such as enters into the composition of Diet 84, is not essential to the production of rickets. In our experiment, although the Ca was reduced to one-fifth of the original amount, marked rachitic lesions still developed. Even with the reduction of the calcium to 0.018 gm. per 100 gm. of diet the lesions, as has been stated, were those of an atypical rickets rather than an osteoporosis. This is what one might expect, in view of the fact that, within wide limits, the blood Ca is independent of the Ca intake.

In reviewing the literature for comparable experiments, we should mention first the studies of the Johns Hopkins investigators. Their fundamental observation that rachitic changes may be regularly induced on a diet deficient in phosphate ion, and that the introduction of the phosphate ion into the diet prevents the rickets, is in accord with our own work. A quantitative comparison from the point of view of the phosphorus content of the diet is somewhat difficult, because it is not clearly stated in their papers which diets led to rickets and which to osteoporosis. In their third paper (5) two diets are given, one (Lot 2667) containing 0.2165 gm. of phosphorus per 100 gm. of diet, the other (Lot 2806), 0.1580 gm. On these two diets the rats showed lesions ranging from simple osteoporosis to florid rickets, but it is not stated whether the more typically rachitic rats were those on the diet containing the lower amount of phosphorus. In our experience Diet 2806 would have been on the border-line; Diet 2667 would lead only to osteoporotic changes. A third diet (Lots 3120 and 2815) contained 0.5383 gm. of phosphorus, and on this the lesions were typically osteoporotic.

In Paper V (6) another rickets-producing diet is given (Ration 3127) in which the phosphorus content is stated to be 0.209 gm. per 100 gm. of diet. This is decidedly above the amount which we have found to give protection. The discrepancy may be due to the form in which the phosphorus was given, to differences in the amount of roughage, or to factors as yet undetermined.[1]

[1] Since this paper was submitted, Eddy, Muller, and Heft (Eddy, W. H., Muller, H. R., and Heft, H. L., *J. Biol. Chem.*, 1922, L, p. xix) have presented experiments indicating that phytin phosphorus added to Diet 84 in double the amount supplied by the inorganic phosphate of Diet 85 fails to prevent rickets. In the diets used by McCollum and his coworkers, a large proportion of the total phosphorus must be assumed to be present in the form of phytin phosphorus. This may account for the discrepancy as regards the minimal amount of phosphorus required to protect against rickets.

A recent paper by Korenchevsky (11) also affords an interesting comparison. Negative results were obtained in phosphate deficiency experiments, but these may be explained, as the author himself points out, by the relatively high phosphorus content (0.220 gm.) of his basic diet, which is well above our protective level of 0.160 gm. per 100 gm.

While our quantitative figures obviously have no claim to finality, since the actual phosphorus intake will vary with the character of the diet and the general condition of the individual rat, we believe from our experience that it is possible to approximate very closely the phosphorus requirement for normal calcification, when the experimental conditions as regards light, age, rate of growth, and type of diet are standardized.

The question has been raised as to whether it is justifiable to apply the term experimental rickets to a condition which seems as yet unrelated etiologically to the human disease. We believe so, and for several reasons. The term rickets is habitually used in an anatomical sense, as referring to certain well characterized changes in the bones. That such alterations may be produced in various ways, and that factors other than dietary enter into the causation of human rickets may be freely admitted. But these considerations, it would seem, should not affect the propriety of terming the experimentally produced lesions rachitic, provided they conform accurately to the lesions which characterize the human disease.

Furthermore, the application of the facts observed in these experiments may not be so alien to the etiology of human rickets as would seem on first thought. A gross deficiency of calcium or phosphorus does not, it is true, occur under ordinary conditions; and rickets commonly develops in infants in spite of what appears to be an adequate supply of these inorganic substances in the food. It cannot be said, however, that we possess an accurate knowledge of the phosphorus requirement of the growing child, nor can it be said dogmatically that the amount contained in milk, for example, is in all cases sufficient without the adjuvant action of sunlight. The recent observations of Howland and Kramer (12), and confirmatory studies which will be reported in full elsewhere, indicate that there is, in active human rickets, and in the experimental rickets of rats produced by phosphorus-low diets, a striking deficiency in the inorganic phosphates of the blood. How this

is brought about remains to be investigated, but it has not been disproved that the proximate cause of rickets, in infants as well as in rats, lies in an inadequate supply of phosphate to the bones.

CONCLUSIONS.

1. Rachitic bone lesions may be produced in rats by a diet containing an excess of calcium, but deficient in phosphates.

2. Similar lesions follow a diet deficient in calcium, but containing an excess of phosphates. The endochondral lesions, however, are less pronounced.

3. A diet deficient in both calcium and phosphate induces atypical rickets.

4. Inorganic salts other than calcium or phosphate seem to be without influence upon the development or prevention of rachitic lesions.

5. The minimal amount of phosphate (calculated as P) required for normal calcification in young growing rats (30 to 50 gm.) on the diets used in these experiments is approximately 160 mg. per 100 gm. of diet. At 135 mg. individual variations come into play; at 110 mg., or less, rickets invariably follows.

6. An excess of calcium is not necessary to the production of the rachitic lesions. A reduction of the calcium lactate of Diet 84 to 0.6 per cent (111 mg. of Ca per 100 gm. of diet) still results in the development of typical rickets.

7. Adult rats react to a deficiency of phosphate in the diet by the production of calcium-free osteoid in abnormal amount about the spongiosa and cortex. There is not produced any alteration of the epiphyseal cartilage, as in growing animals.

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EXPLANATION OF PLATES.

PLATE 21.

FIG. 1. Rat 82. 52 days on Eiweissmilch. Normal growth. Normal rib.

FIG. 2. Rat 90. Diet 84 plus 0.8 per cent secondary *sodium* phosphate. Radiograph showing normal bones on 35th day.

FIG. 3. Rat 89. 35 days on Diet 84 plus 0.8 per cent secondary *sodium* phosphate. Rib: The zone of proliferating cartilage is about four cells deep, the matrix between the columns is calcified. The primary spongiosa is very defective, and epiphyseal growth in abeyance. Secondary spongiosa also scant. Cortex narrow; an endosteal osteoid margin bordered by osteoblasts is visible in a few places, but is not wider than in normal bones of young rats. No rickets. 11 days decalcification in Müller's fluid. Hematoxylin-eosin stain.

PLATE 22.

FIG. 4. Rat 97. 25 days on Diet 84 plus 0.35 per cent *potassium chloride*. Radiograph showing marked rickets.

FIG. 5. Rat 99. 36 days on Diet 84 plus 0.35 per cent *potassium chloride*. Rib: Zone of proliferating cartilage greatly widened with deep prolongations. Matrix uncalcified. Spongiosa represented by dense mass of Ca-free osteoid, with narrow Haversian spaces between the broad and convoluted trabeculæ. A mass of osteoid fills in the angular depression on the external surface of the chondrocostal junction. Wide osteoid margin about cortex. Perichondrium and periosteum thickened. Marked rickets. 11 days decalcification in Müller's fluid. Hematoxylin-eosin stain.

PLATE 23.

FIG. 6. Rat 117. 34 days on Diet 85N (Diet 84 with the addition of basic potassium phosphate, equivalent to 50 mg. of P per 100 gm. of diet). Slight or early rachitic lesions (see text). 6 days decalcification in Müller's fluid. Silver nitrate-Van Gieson stain.

FIG. 7. Rat 119. 34 days on Diet 85O (Diet 84 with the addition of basic potassium phosphate equivalent to 25 mg. of P per 100 gm. of diet). More marked rachitic lesions than on Diet 85N. 6 days decalcification in Müller's fluid. Silver nitrate-Van Gieson stain.

PLATE 24.

FIG. 8. Rat 168. 26 days on Diet 84 plus Osborne-Mendel salt mixture without phosphoric acid. Radiograph showing marked rickets.

FIG. 9. Rat 392. 29 days on Diet 85C. The zone of preparatory calcification averages ten to twelve cells, not regularly aligned in columns. There are no deep projections into the metaphysis. Calcium is laid down between the distal three or four cells, but is defective in the central portion. The spongiosa is composed of broad trabeculæ of osteoid, with a narrow central core of calcified cartilage or bone. The perichondral osteoid is calcium-free and slightly broader than normally. The calcified cortex is thin, and covered on both sides by a broad margin of osteoid. There is irregular fibrous periosteal thickening. The margin between calcified bone and osteoid is everywhere sharp. The lesion differs from early rickets only in the relatively slight alterations of the cartilage.

FIG. 10. Rat 493. 26 days on Diet Q. Zone of preparatory calcification eight to ten cells in depth and entirely calcium-free. Much subchondral and cortical osteoid. The apparently calcified bone stains faintly with hematoxylin, and gives no reaction with silver nitrate. 5 days decalcification in Müller's fluid.

FIG. 1.

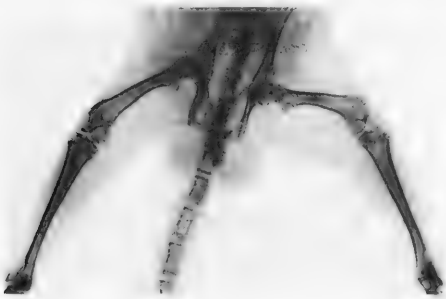


FIG. 2.

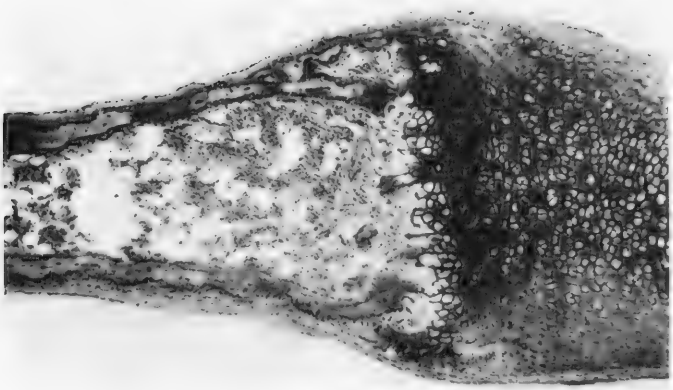
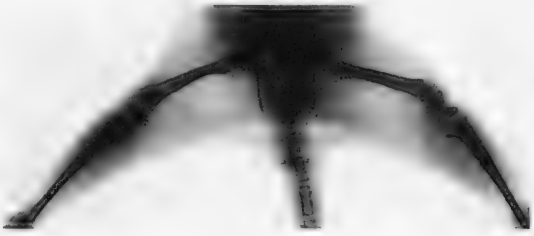


FIG. 3.

(Pappenheimer, McCann, and Zucker: Experimental rickets. IV.)



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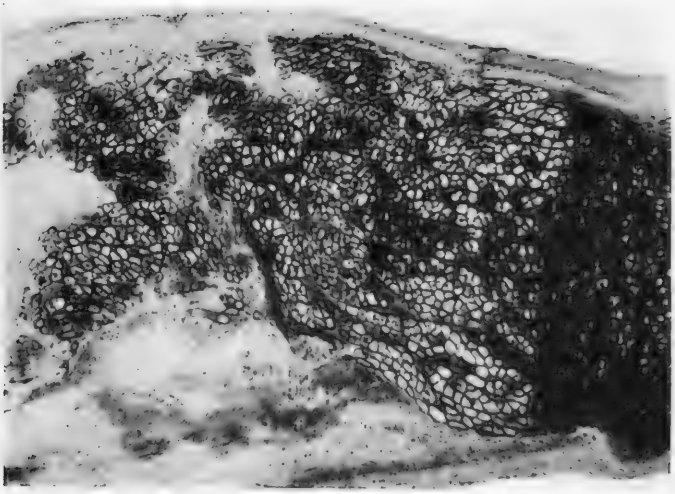

FIG. 5.

(Pappenheimer, McCann, and Zucker: Experimental rickets. IV.)

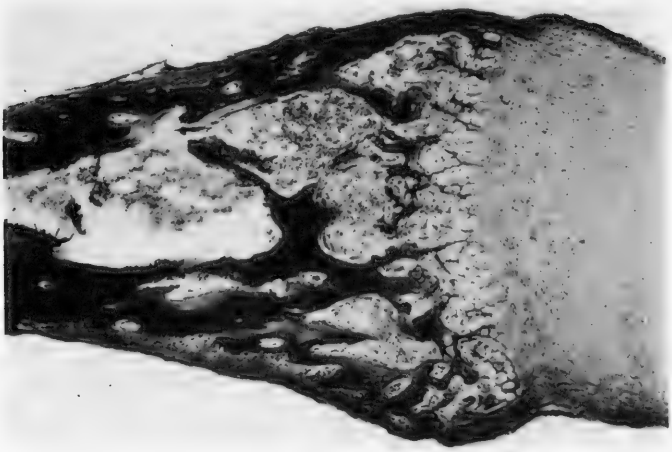

FIG. 6.

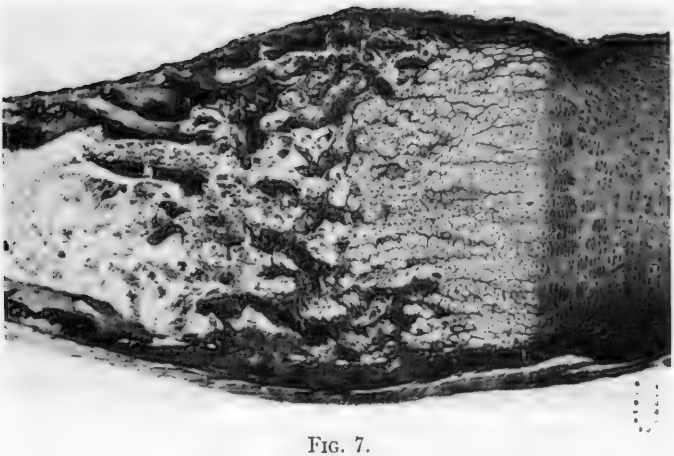

FIG. 7.

(Pappenheimer, McCann, and Zucker: Experimental rickets. IV.)

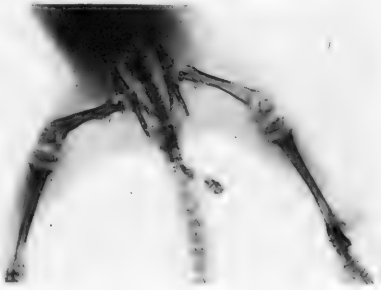

FIG. 8.

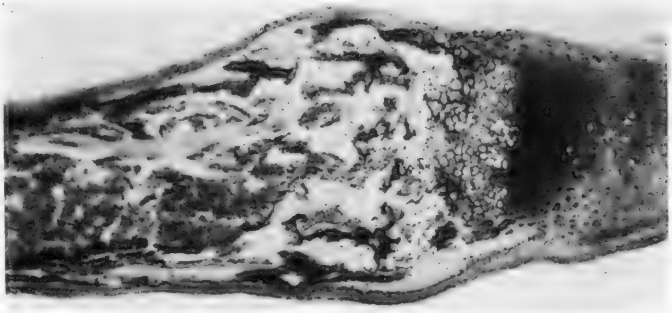

FIG. 9.

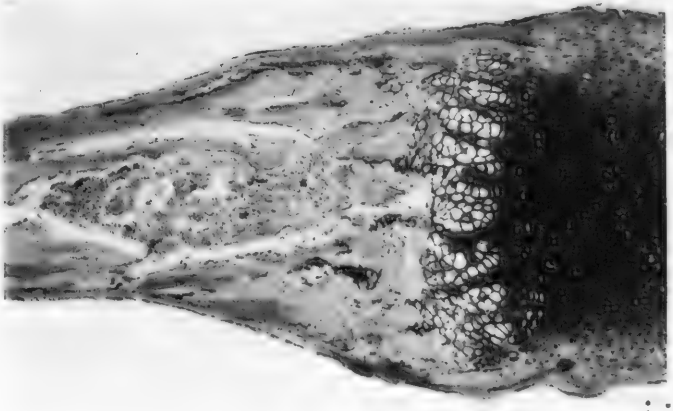

FIG. 10.

(Pappenheimer, McCann, and Zucker: Experimental rickets. IV.)

EXPERIMENTAL RICKETS IN RATS.

V. The Effect of Varying the Organic Constituents of a Rickets-Producing Diet.*

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Plates 25 to 29.

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In a preceding article (1) it was shown that rachitic lesions may be regularly induced in young growing rats by a deficiency of PO_4 in the diet, provided the Ca ion is present in sufficient quantity; and that a deficiency of both Ca and PO_4, or of Ca alone leads to bone changes which differ in certain respects from those of typical rickets.

The present study is concerned with the bone changes which result from modifying the organic constituents of the rickets-producing diet. We have sought, in particular, an answer to the following questions.

1. Is phosphorus, ingested in organic combination, of equal value, as compared with inorganic phosphorus, for the purposes of bone formation?

2. Do the water-soluble and fat-soluble vitamines exert any protective or curative effect, apart from the phosphorus content of the substances in which they occur? It has already been shown that the rachitic lesions which follow a diet lacking in phosphates may be prevented by merely remedying this deficiency (2), and that rats maintained throughout their growth period upon a diet deficient in fat-soluble vitamine, but adequate as regards salt content, fail to become rachitic (3). It was still conceivable that the addition of the fat-soluble factor to the rachitic diet might exercise some protec-

* A preliminary report of this work was presented before the Society of Experimental Biology and Medicine (1).

tive influence. A possible influence of the water-soluble factor also remained to be investigated.

3. Is it possible to make the rickets-producing Diet 84 more nearly adequate for proper nutrition and growth, without impairing its rickets-producing effect? Rachitic infants are often well nourished, whereas Diet 84, as has been pointed out, is not adequate for the normal growth and nutrition of rats. It was an obvious problem to attempt to bridge this discrepancy in amplifying Diet 84 by the addition of the lacking food constituents.

The experimental procedure has been fully detailed in the preceding paper, and need not be again described.

Effect of the Addition of Casein to Diet 84.

In order to study the effect of phosphorus in the form of phosphoprotein, 10 per cent of casein was substituted for the same percentage of flour in Diet 84. The casein used was the ordinary commercial product which had been extracted with cold alcohol by slow percolation until the washings were colorless, and then extracted for 48 hours with two changes of ether. Analysis of this casein showed a P content of 0.840 per cent. The diet contained approximately the same amount of phosphorus (162 mg. per 100 gm.) as Diet 85.

The results of this experiment, as shown in Table I, require further comment. Rat 95, which showed moderate rickets on x-ray examination, was killed on the 22nd day (Fig. 1). Gross examination showed a slight beading and haziness of the chondrocostal junctions. Microscopically, there were found rachitic lesions of moderate intensity (Fig. 2).

Rat 95.—Rib: The zone of proliferative cartilage is very broad, averaging twenty-five to thirty cells in depth. From this, single or double rows of cartilage cells extend into the metaphysis, and between these the matrix is calcified. No demonstrable calcium, however, is present in the proximal or basal portion of the zone of preparatory calcification. There is a very dense spongiosa composed for the most part of uncalcified osteoid tissue. A few of the trabeculæ show an inner ossified tissue enveloping the calcified cartilaginous matrix. The osteoid tissue is more than normally abundant along the cortex, especially about the perforating vessels. The marrow space of the shaft is broad, the marrow cellular. The lesion may be interpreted as early healing of a moderate rickets.

The other two rats of this series (Nos. 104 and 105) showed in radiographs taken on the 22nd day (Fig. 3) slight but definite changes which were interpreted as rachitic. Radiographs taken on the 36th day, however, showed apparently complete healing (Fig. 4). No rachitic deformities were found at autopsy, nor did microscopic study of the ribs show evidence of the previous lesion (Fig. 5).

These experiments strongly suggested that the casein phosphorus conferred a less complete protection than would be given by an equivalent amount of phosphorus in the form of basic potassium phosphate. The following experiments confirm this. Three rats (Nos. 292 to 294) were placed on Diet 84 with 5 per cent purified casein substituted for an equal percentage of flour. After 28 days all three rats showed, by radiograph, marked rickets; and one rat sacrificed at this time showed severe deformities and characteristic microscopic lesions without healing. Three other rats (Nos. 295 to 297) were given 15 per cent of casein, equivalent to a total P content of 200 mg. per 100 gm. of diet—an amount considerably in excess of that required to afford complete protection when administered as inorganic phosphate. Of these, all showed by x-ray examination on the 28th day moderate rickets. In one rat (No. 296), however, healing was apparently in progress as judged by the presence of a definite transverse shadow in the head of the tibia. Another rat (No. 297) killed on this day and examined microscopically proved to have moderate rachitic lesions with partial healing. The third rat of this group (No. 295) showed no healing radiographically on the 37th day. It was found dead in the cage and too decomposed for histological study.

The only conclusion that can be drawn from this series is that the protection afforded by casein during the period of most active growth is not wholly equivalent to that given by the same amount of phosphorus in the form of basic potassium phosphate. We have as yet no explanation for the discrepancies shown by individual rats. The difference cannot be correlated merely with the differences in the rate of growth.

Effect of the Addition of Lecithin to Diet 84.

Lecithin was added to the diet with the view of ascertaining whether phosphorus in the form of a phospholipin exerts an equivalent pro-

TABLE I.

Effect of the Addition of Casein to Diet 84.

Rat No.	Litter No.	Diet.	Length of time on diet.	Weight.			Radiograph.		Pathological examination.		
				Initial.	Maximum.	Final.	Day of diet.	Rickets.	Rickets.	Ophthalmia.	Other lesions.
			days	*gm.*	*gm.*	*gm.*					
		No. 84 + casein.									
95	15	Flour.....85.0 per cent. Casein.....10.0 " " Calcium lactate.... 2.9 " "	22	38	50	50	22nd	Moderate.	Moderate.*	None.*	None.
104	17	Sodium chloride.... 2.0 " " Ferric citrate..... 0.1 " "	38	31	48	48	22nd 36th	Slight (?). None.	None.*	" *	"
105	17		38	31	47	44	22nd	Moderate.	" *	"	"
		No. 84 + casein.									
292		Flour.....90.0 per cent.	44	31	43	39	28th	Marked.	Healing.*	None.*	
293		Casein..... 5.0 " " Calcium lactate.... 2.9 " "	28	42	60	60	28th	"	Marked.*	"	None.
294		Sodium chloride.... 2.0 " " Ferric citrate..... 0.1 " "	44	37	52	52	28th	"	Healing.*	" *	
		No. 84 + casein.									
295		Flour.....80.0 per cent.	44	37	43	43	28th	Marked.	?		
296		Casein.....15.0 " " Calcium lactate.... 2.9 " " Sodium chloride.... 2.0 " "	44	37	46	46	28th	" healing.	Healed.*		
297		Ferric citrate..... 0.1 " "	28	54	80	80	28th	Moderate.	Slight, healing.*	None.	None.

Controls from the same litters.												
		No. 84.										
92	15	Flour.....95.0 per cent.	44	32	42	32	27th	Moderate.	Marked.*	Panophthalmitis.	Atelectasis.	
		Calcium lactate.... 2.9 " "					34th	Marked.				
94†	15	Sodium chloride.... 2.0 " "	45	29	44	44	27th	Moderate.	Cured with cod liver oil.			
		Ferric citrate..... 0.1 " "					34th	Marked.				
102†	17		35	43	53	53	25th	Moderate.	" "	" "		
							38th	Marked.				
103†	17		44	28	43	34	25th	Moderate.				
							38th	Very marked.	" "	" "		

* In all the tables the asterisk indicates that the finding was confirmed by microscopic examination.

† Rickets healed by cod liver oil, 5 drops every day, given for 5, 15, and 10 days respectively.

TABLE II.

Effect of the Addition of Lecithin to Diet 84.

Rat No.	Litter No.	Diet.	Length of time on diet.	Weight.			Radiograph.		Pathological examination.		
				Initial.	Maximum.	Final.	Day of diet.	Rickets.	Rickets.	Ophthalmia.	Other lesions.
			days	*gm.*	*gm.*	*gm.*					
		No. 84 + lecithin.									
145	25	Flour.....93.0 per cent.	30	34	43	41	28th	None.	None.*	None.	None.
		Lecithin..... 2.0 " "									
146	25	Calcium lactate.... 2.9 " "	30	33	49	49	28th	"	" *	"	"
		Sodium chloride ... 2.0 " "									
147	25	Ferric citrate..... 0.1 " "	30	36	51	51	28th	"	" *	"	"

tection to that given by inorganic phosphate. Three rats (Table II, Nos. 145 to 147) were given a diet similar to Diet 84, except that 2 per cent of the flour was replaced by an equal weight of lecithin (commercial product). The lecithin was dissolved in ether and mixed with the flour in this way, thus ensuring an even distribution of the lecithin throughout the mixture. The ether was then allowed to evaporate at room temperature, and the salt mixture added. The 2 per cent of lecithin was calculated to contain about 79 mg. of phosphorus, making a diet which was comparable in its phosphorus content to Diet 85. The total phosphorus of the diet was found by analysis in duplicate to be 152.4 and 153.2 mg. per 100 gm. of diet.

After 30 days on this diet the rats showed no evidence of rickets either by x-ray examination or microscopically (Figs. 6 and 7). The conclusion is evident, therefore, that phosphorus in the form of lecithin afforded protection equal to that given by inorganic phosphates.

Effect of the Addition of Yeast to Diet 84.

Yeast extract was added to the diet with the double purpose of ascertaining the influence of the water-soluble vitamine and of another phosphorus-containing substance. The preparation used was Harris' yeast vitamine (Osborne and Wakeman (4)) which we found to contain 42 mg. of phosphorus per gm.

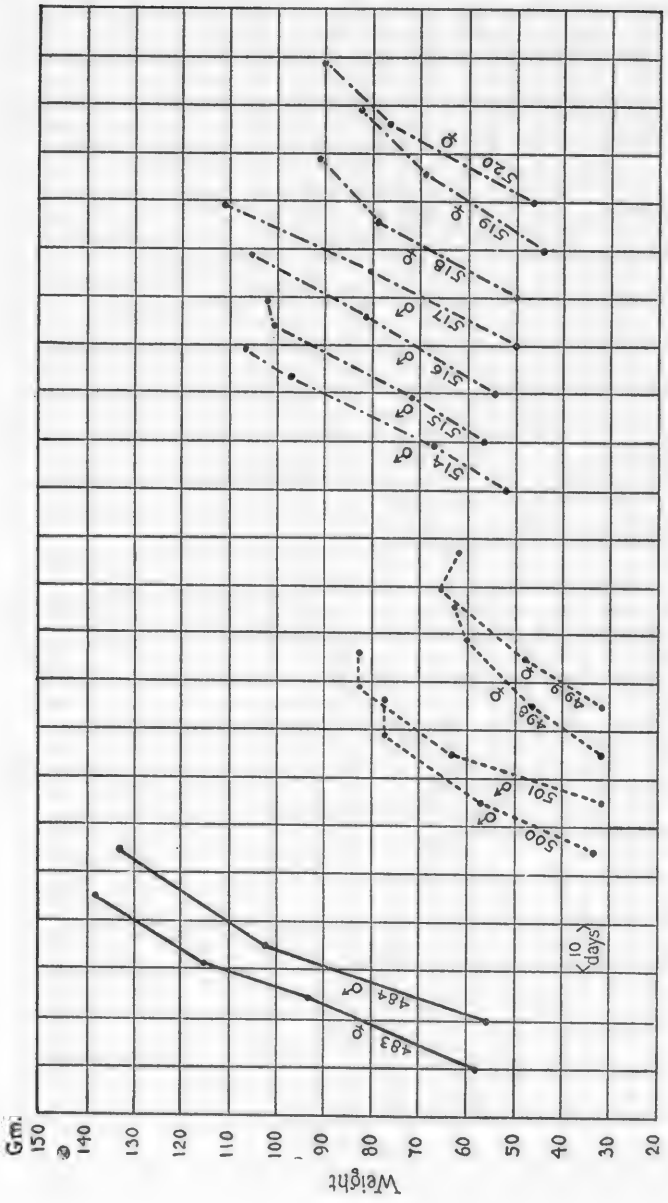
In a preliminary experiment about 100 mg. of the extract were measured roughly and sprinkled over each feeding. The rats always ate the yeast avidly as soon as it was placed in the cage. The three rats used for this test (Table III, Nos. 96, 108, and 109) showed no rickets on x-ray examination or section, after 37, 35, and 37 days respectively on the diet.

At the time of the experiment, it was not realized that the phosphorus content of this yeast preparation was as great as it was subsequently shown to be. Therefore, it was necessary to reduce the amount of the yeast extract so as to bring the phosphorus content to, and below the level of protection, and yet supply an adequate amount of water-soluble factor. Data for determining this were available from the work of Osborne and Wakeman (4), who showed that 20 mg. of such a yeast preparation carried ample vitamine to ensure proper growth when the other food constituents were present in

TABLE III.

Effect of the Addition of Yeast Extract to Diet 84.

Rat No.	Litter No.	Diet.	Length of time on diet.	Weight.			Radiograph.		Pathological examination.		
				Initial.	Maximum.	Final.	Day of diet.	Rickets.	Rickets.	Ophthalmia.	Other lesions.
			days	*gm.*	*gm.*	*gm.*					
		No. 84 + yeast.									
96	15	(100(?) mg. of Harris' yeast vitamine given daily to rats on basal Diet 84.)	37	37	60	60	22nd	None.	None.*	Slight.	None.
108	17		35	33	53	52	22nd	"	" *	Panophthalmitis (right); corneal opacity (left).	"
109	17		37	29	50	50	22nd	"	" *	Moderate keratitis.*	"
166	28	No. 84+0.5 per cent yeast extract containing 21 mg. of phosphorus per 100 gm. of diet.	26	36	46	46	25th	Moderate.	Slight.*	None.	None.
171	29		26	40	50	50	25th	Marked.	See Table VI.		
165	28	No. 84+1.25 per cent yeast extract containing 52 mg. of phosphorus per 100 gm. of diet.	26	33	41	41	25th	Moderate.	Slight.*	None.	None.
167	28		26	28	50	50	25th	"	See Table VI.		



——Controls on full diet. .....Rats on Diet D. —·—·— Rats on Diet E.

TEXT-FIG. 1. Growth of rats on Diets D and E compared with that of controls on a full diet.

adequate amount. There has since accumulated additional evidence that the water-soluble B content of our diets is sufficient to permit of normal growth during the experimental period when the other deficiencies are rectified. Rats on Diets D and E, in which no additional water-soluble vitamine is added, have shown growth closely approximating the normal, at the same time that they were developing rickets (Text-fig. 1). These diets will be discussed under the section on amplified diets.

Two groups were given diets in which 0.5 per cent (Rats 166 and 171) and 1.25 per cent (Rats 165 and 167) of yeast extract replaced equal weights of the flour in Diet 84. In these experiments the extract was mixed evenly through the diet, and not added separately as before. 0.5 per cent of the yeast vitamine was calculated to give 25 mg. of extract per day (the rats each ate about 5 gm. of the total diet per day) and added 21 mg. of phosphorus per 100 gm. of diet. 1.25 per cent gave 62 mg. of extract per day, and 52 mg. of phosphorus per 100 gm. of diet. On these diets all the rats developed marked rickets, as shown by radiograph and section (Figs. 8 and 9).

This would indicate that the water-soluble vitamine may be present in the diet in large amount without any protective effect. A larger indefinite amount of this preparation protected, but here the phosphorus added was sufficient to account for the protection.

Effect of the Addition of Egg Albumin to Diet 84.

The phosphoprotein casein having been shown to afford at least a partial protection, probably dependent upon its phosphorus content, we attempted to improve the nutrition by the addition of a phosphorus-free protein. The phosphorus content of the egg albumin used was negligible, 10 gm. samples not yielding enough ammonium phosphomolybdate precipitate to titrate. 10 per cent of egg albumin (commercial product) was substituted in Diet 84 for an equivalent amount of flour. This addition alone did not strikingly improve the general nutrition. There was also no protective effect. Rat 159 (Table IV) kept on this diet for 26 days showed rickets by radiograph (Fig. 10) and at autopsy (Fig. 11). Rats 160 and 161, also on this diet for 26 days, showed even more severe rickets radiographically and were subsequently used for other work.

TABLE IV.

Effect of the Addition of Egg Albumin to Diet 84.

Rat No.	Litter No.	Diet.	Length of time on diet.	Weight.			Radiograph.		Pathological examination.		
				Initial.	Maximum.	Final.	Day of diet.	Rickets.	Rickets.	Ophthalmia.	Other lesions.
			days	*gm.*	*gm.*	*gm.*					
		No. 84 + egg albumin.									
159	26	Flour..... 85.0 per cent. Egg albumin.....10.0 " " Calcium lactate..... 2.9 " "	26	33	43	43	24th	Moderate.	Marked.*	None.	None.
160	26	Sodium chloride ..... 2.0 " " Ferric citrate..... 0.1 " "	26	44	50	50	24th	Marked.	See Table VI.		
161	26		24	40	61	61	24th	"	" " VI.		

Effect of the Addition of Butter and of Butter Fat to Diet 84.

The addition of butter was of particular interest in view of the much discussed rôle of the fat-soluble vitamine in the prevention of rickets.

The conception of rickets as a vitamine deficiency disease was first propounded by Funk (5). The views and experiments of Mellanby (6, 7) in support of this theory have been so widely quoted that no further reference need be made to them.

Subsequent work in general has been unfavorable to Mellanby's conclusion. Harden and Zilva (8) in monkeys, Hess and Unger (9) in infants, Paton and Watson (10) in dogs, Hess, McCann, and Pappenheimer (11) in rats, Mackay (12) and Tozer (13) in kittens, Tozer (14) in guinea pigs, Zilva, Golding, Drummond, and Coward (15) in pigs have all failed to produce typical rachitic lesions on diets deficient in fat-soluble vitamine.

Shipley, Park, McCollum, and Simmonds (16) cite experiments in which a diet adequate in phosphorus, but deficient in fat-soluble A failed to produce rickets. In subsequent papers (17, 18) these authors state that the addition of 10 per cent of butter fat to a rickets-producing diet is ineffective in preventing the development of rachitic lesions, although 3 per cent of the butter-fat carried sufficient fat-soluble vitamine for the promotion of growth and fertility.

The latest publication on this phase of the subject is that of Korenchevsky (19), who concludes that ordinarily a deficiency in fat-soluble A produces in young rats merely an osteoporosis with deficient osteogenesis, but in some cases, namely, when the parents during conception, pregnancy, and lactation have been fed on a fat-soluble-deficient diet, changes more analogous to rickets are produced in the offspring. He concludes, therefore, that vitamine A has a relation to the metabolism of calcium and to the development of rickets.

In our first experiment four rats (Nos. 73, 74, 79, and 80, Table V) were given Diet 84 modified by the substitution of 5 per cent of pasteurized butter for flour. This gave about 0.20 to 0.25 gm. of butter per rat per day. This amount of butter of the same brand completely protected all control animals against keratomalacia and evoked a characteristic rise in the weight curve with cure of keratomalacia in two rats which had been maintained for a long time on a fat-soluble-deficient diet (11). The rats on Diet 84 plus 5 per cent butter, after 32, 43, 45, and 45 days respectively, were killed and found to show marked rickets (Figs. 12 and 13).

Incidentally, it may be mentioned that eye lesions occurred with extreme frequency (over 90 per cent), irrespective of the presence

TABLE V.

Effect of the Addition of Butter to Diet 84.

Rat No.	Litter No.	Diet.	Length of time on diet.	Weight. Initial.	Weight. Maximum.	Weight. Final.	Radiograph. Day of diet.	Radiograph. Rickets.	Pathological examination. Rickets.	Pathological examination. Ophthalmia.	Pathological examination. Other lesions.
			days	*gm.*	*gm.*	*gm.*					
		No. 84 + butter.									
73	12	(0.2 gm. of butter pasteurized, given daily to rats on basal Diet 84.)	43	24	40	40	16th	None.			
							23rd	Slight(?).	Marked.*	None.*	None.
74	12		45	28	46	46	16th	None.			
							23rd	"	" *	" *	"
							44th	Marked.			
79	13		32	33	49	49	31st	Slight.	Moderate.*	"	"
80	13		45	33	52	52	44th	Moderate.	Marked.*	" *	"
		No. 84 + butter.									
142	25	(0.4 gm. of fresh butter given daily to	30	41	43	41	27th	Negative.	Slight.*	None.	"
143	25	rats on Diet 84.)	28	35	41	41	27th	"	" *	"	"
144	25		30	40	42	39	27th	"	" *	"	"
Controls from the same litters.											
		No. 84.									
71	12	Flour.....95.0 per cent.	41	28	42	33	28th	Moderate.	Marked.*		Atelectasis.
							34th	Marked.			
		Calcium lactate..... 2.9 " "									
72	12	Sodium chloride..... 2.0 " "	34	26	37	37	24th	Moderate.	" *	Slight.	
		Ferric citrate..... 0.1 " "									

or absence of rachitic lesions, in rats on Diets 84 and 85, and the modifications described.

In spite of certain work indicating that the fat-soluble vitamine is fairly resistant to heat (Osborne and Mendel (20)), it seemed advisable to make certain that we were giving an excess of this factor by repeating the experiments, using butter which had not been subjected to heat.

To a second series of rats (Nos. 142 to 144, Table V) 0.4 gm. per rat of fresh butter, made every few days in the laboratory from fresh cream, was given daily. This butter contained 20 per cent of whey, and on analysis 2.4 mg. of phosphorus per gm. The 0.9 mg. of phosphorus given daily was thus far below the amount necessary to protect, as shown in the previous experiments. After 28, 30, and 30 days on this diet (Fig. 14) these three rats were killed, and all showed rickets of moderate degree microscopically. While the lesions were not so extreme as those which had been observed in some rats on other diets for a corresponding period, they were quite definite, and there was no evidence of healing or protection.

Moreover, butter fat, though given in amounts carrying ample fat-soluble vitamine, seems to have no curative effect upon rickets already established. Osborne and Mendel (20) found that 6 per cent butter fat carried enough for adequate growth and the prevention of eye lesions. In our experiments 10 per cent of two butter fats, one made from fresh raw (farm) butter and one from the same brand of pasteurized butter as used before, was substituted for an equivalent amount of flour in an amplified diet described below. Rachitic rats Nos. 161 and 190 were given the raw butter fat, and rachitic rats Nos. 160, 167, 169, and 171 (Table VI) the pasteurized butter fat. After 8 to 10 days on this diet these rats still showed marked rickets with no evidence of healing in spite of excellent general health.

The foregoing experiments seem to us to give convincing evidence that the fat-soluble vitamine, as usually defined (see, for example, Steenbock, Sell, and Buell (21)), plays no part in the pathogenesis of rat rickets.

Effect of the Addition of Meat to Diet 84 (Meat and Flour Diet).

Three rats (Nos. 281 to 283) were fed for periods of 33, 39, and 33 days on Diet 84, supplemented by the addition of chopped and dried round steak *ad libitum*. The growth on this diet was normal, and examination of the bones radiographically and microscopically showed a normal structure.

This diet was adequate in both calcium and phosphorus.

Three rats (Nos. 284 to 286) were placed for a period of 35 days upon a diet of dried chopped steak and patent flour, without the addition of sodium chloride, calcium lactate, or ferric citrate. This gave a diet rich in phosphorus, but deficient in calcium. Growth on this diet was poor. The lesions resulting in the ribs resemble rather closely those described in the previous paper on Diet 85C—low in calcium, but containing an excess of phosphate—namely, slight irregularity and widening of the zone of preparatory calcification, defective calcium deposition in the cartilage, and slight excess of osteoid. Marked rachitic deformities were not produced (Fig. 15).

It is interesting to compare these findings with those of Watson (22) in rats maintained on an exclusive meat diet. Watson's rats showed deformities and infractions resembling those of rickets, but microscopically the epiphyseal changes were very slight.

Amplified Rachitic Diet.

In the first paper of this series (2) it was pointed out that Diet 84 is deficient in the character of the protein, in its fat-soluble vitamine content, and in various inorganic constituents. The following diets were devised to remedy these deficiencies.

In a preliminary series rats already rachitic, as shown by radiograph, were placed on Diets 84R, $84R_1$, and $84R_2$ (Table VI), in which Diet 84 is enriched by the substitution of a more complete protein (egg albumin), butter or butter fat, and a salt mixture complete in every respect except phosphorus (Osborne and Mendel (23)). No tendency to healing of the bone lesions was shown microscopically after 8 to 10 days on this diet. Although the general condition of the animals was greatly improved during this time, as shown by sleekness and amount of body fat, the weight was not greatly increased.

TABLE VI.

Amplified Diets.

Rat No.	Litter No.	Previous diet.	Length of time on previous diet.	Rickets as shown by radiograph.	Amplified diet.						Pathological examination.		
					Diet.	Length of time on diet.	Weight.		Radiograph.		Rickets.	Ophthalmia.	Other lesions.
							Initial.	Final.	Day of diet.	Rickets.			
			days			*days*	*gm.*	*gm.*					
160	26	No. 84 + egg albumin.	26	Marked.	No. 84R.	11	50	51	9th	Marked.	Marked.*	None.	None.
161	26	No. 84 + egg albumin.	24	"	" 84R.	10	59	60	9th	"	Very extreme.*	"	"
					" $84R_2$.	11							
167	28	No. 84 + 1.25 per cent yeast extract.	26	Moderate.	" $84R_1$.	8	45	50			Very extreme.*	"	"
169	29	No. 84 with Osborne-Mendel salt mixture.	26	"	" $84R_1$.	8	26	30			Marked.*	"	"
171	29	No. 84 + 0.5 per cent yeast extract.	26	Marked.	" $84R_1$.	8	49	50			" *	"	"
190		No. 84.	28	Moderate.	" $84R_2$.	9	93	96			Very extreme.*	"	"

Diet 84R.

Flour	78.0 per cent.
Osborne-Mendel salt (phosphorus-free)	5.0 " "
Calcium lactate	2.0 " "
Egg albumin	10.0 " "
Butter (pasteurized)	5.0 " "

Diet $84R_1$.

Flour	73.0 per cent.
Osborne-Mendel salt mixture (phosphorus-free)	5.0 " "
Calcium lactate	2.0 " "
Egg albumin	10.0 " "
Butter fat (pasteurized)	10.0 " "

Diet $84R_2$.

Like Diet $84R_1$ except that raw butter fat was used.

The diet which is being used at present (Diet D) gives a growth curve which, as has been stated, approximates the normal during about 1 month. The composition of the diet is as follows:

Patent flour	80.9 per cent.
Egg albumin	10.0 " "
Butter fat	5.0 " "
Salt mixture	4.1 " "

The salt mixture furnished the following constituents.[1]

KCl	0.85	gm.	per	100	gm.	of diet.
Na_2CO_3	0.85	"	"	100	"	"
$MgCO_3$	0.286	"	"	100	"	"
Ca lactate	2.000	"	"	100	"	"
Ferric citrate	0.1	"	"	100	"	"
KI	0.0002	"	"	100	"	"
$MnSO_4$	0.00078	"	"	100	"	"
NaF	0.0024	"	"	100	"	"
$KAl(SO_4)_2$	0.00024	"	"	100	"	"
Total	4.08962	"	"	100	"	"

This salt mixture is based on analyses of the milk of small animals instead of cow's milk, as in the Osborne and Mendel mixture.

Rachitic lesions develop regularly on this diet (Fig. 16). A few of the rats which failed to grow because of infections have shown less deformity than more actively growing rats. This has been noted on other rickets-producing diets, and is in accordance with clinical experience in children. An obvious explanation may be found in the greater phosphorus needs of all tissues in the rapidly growing animal.

In Diet E, 72 mg. of phosphorus were added to the above diet. Owing to the substitution of 15 per cent of non-phosphorus-containing substance for flour, the total phosphorus content was reduced to 145 mg. per 100 gm. of diet. This is an amount which has been shown to be on the border-line of phosphorus requirement. These rats showed excellent growth (Text-fig. 1). Of thirteen rats on this diet,

[1] The reasons for using this salt mixture will be discussed in a forthcoming paper.

eight showed no rickets, four minimal rickets, and only one typical, but moderate, lesions. It is in accord with the explanation offered that the rachitic changes in some of these rats were more marked than on simpler diets with equivalent phosphorus content.

CONCLUSIONS.

1. Casein phosphorus does not completely prevent the development of rickets when substituted in Diet 84 in amount equivalent to a protective dose of basic potassium phosphate.

2. The protection given by lecithin is equivalent to its phosphorus content.

3. The protection given by yeast is at least proportional to its phosphorus content. An amount carrying sufficient vitamine B to promote growth, but insufficient to provide adequate phosphorus, does not prevent rickets.

4. Vitamine A, in the form of butter or butter fat to the amount of 10 per cent of the diet, neither prevents nor cures rickets.

5. The substitution of 10 per cent of egg albumin in Diet 84 improves the nutrition, but does not prevent rickets.

6. The addition of meat to Diet 84, thereby supplying an abundance of phosphorus, promotes normal growth and normal bone formation. A diet consisting solely of meat and flour is inadequate for proper growth, and leads to changes in the bones comparable with those observed on a diet low in calcium, but rich in phosphorus.

7. A diet has been found which contains the necessary food elements for approximately normal growth, and in which the only known deficiency is phosphorus. This leads regularly to the production of rickets.

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EXPLANATION OF PLATES.

PLATE 25.

FIG. 1. Rat 95. 22 days on Diet 84 with the substitution of 10 per cent purified casein. Radiograph showing moderate rickets.

FIG. 2. Rat 95. 22 days on Diet 84 with the substitution of 10 per cent purified casein. The rib shows moderate rickets; early healing (?) (see text). 8 days decalcification in Müller's fluid. Hematoxylin-eosin stain.

FIG. 3. Rat 105. 22 days on Diet 84 with the substitution of 10 per cent purified casein. Radiograph showing slight rachitic changes in the upper epiphysis of the tibia.

PLATE 26.

FIG. 4. Rat 105. Radiograph showing healing of rachitic lesion after 36 days.

FIG. 5. Rat 105. 38 days on Diet 84 with the substitution of 10 per cent purified casein. The rib shows a zone of provisional calcification, three to four cells deep; matrix calcified. Primary spongiosa composed of five or six short stout trabeculæ completely ossified, without osteoid margin. Secondary spongiosa poorly developed. Cortex without visible osteoid border. Marrow partly

fatty. No rickets. Osteogenesis inactive. 10 days decalcification in Müller's fluid. Hematoxylin-eosin stain.

FIG. 6. Rat 145. Diet 84 with the substitution of 2 per cent lecithin. Radiograph on the 28th day. No rickets.

FIG. 7. Rat 145. 30 days on Diet 84 with the substitution of 2 per cent lecithin. The rib shows a zone of provisional calcification four to five cells deep. Calcium deposition in matrix normal. Primary spongiosa, extremely delicate, trabeculæ missing in places; those present, regular in arrangement and well calcified. Cortex thinned in subchondral region, elsewhere well developed. No excess of osteoid. Slight osteoporosis. No rickets. 5 days decalcification in Müller's fluid. Hematoxylin-eosin stain.

PLATE 27.

FIG. 8. Rat 166. Diet 84 with the substitution of 0.5 per cent Harris' yeast extract. Radiograph taken on the 25th day. Marked rickets.

FIG. 9. Rat 166. 26 days on Diet 84 with the substitution of 0.5 per cent Harris yeast extract. The rib shows moderate rachitis. Zone of preparatory calcification twenty to thirty cells deep; calcium deposition limited to distal half of cartilage matrix and its prolongations. Dense spongiosa with great excess of osteoid; osteoblasts conspicuous. Osteoid margin about cortex much increased. 3 days decalcification in Müller's fluid. Silver nitrate-Van Gieson stain.

FIG. 10. Rat 159. 24 days on Diet 84 with the substitution of 10 per cent egg albumin. Radiograph showing moderate rickets.

FIG. 11. Rat 159. 26 days on Diet 84 with the substitution of 10 per cent egg albumin. The rib shows typical marked rickets. 9 days decalcification in Müller's fluid. Hematoxylin-eosin stain.

PLATE 28.

Fig. 12. Rat 79. 32 days on Diet 84 plus 5 per cent pasteurized butter. The rib shows definite rachitic lesions. The zone of provisional calcification projects irregularly and is greatly widened. Calcium deposition defective. Spongiosa dense, composed almost wholly of osteoid with inclusions of cartilage cells. Cortex surrounded by wide osteoid border. Marrow spaces reduced. 2 days decalcification in Müller's fluid. Hematoxylin-eosin stain.

FIG. 13. Rat 80. 45 days on Diet 84 plus 5 per cent pasteurized butter. The rib shows advanced rachitic changes at the chondrocostal junction. 3 days decalcification in Müller's fluid. Hematoxylin-eosin stain.

PLATE 29.

FIG. 14. Rat 144. 30 days on Diet 84 plus 0.4 gm. daily of fresh, unpasteurized butter. The rib shows a zone of preparatory calcification twelve to sixteen cells deep, with several prolongations into the metaphysis. Matrix calcium-free,

except for the central core of spongiosa. Trabeculæ of spongiosa broad and abundant, almost wholly osteoid, bordered by conspicuous osteoblasts; osteoid border of cortex increased along entire length of shaft. Moderate rickets. 3 days decalcification in Müller's fluid. Hematoxylin-eosin stain.

FIG. 15. Rat 285. 35 days on meat and flour diet. Zone of preparatory calcification very slightly increased. Calcium deposition limited to matrix between distal two or three cells. Osteoid increased about spongiosa and cortex.

FIG. 16. Rat 498. 26 days on Diet D. Radiograph showing rickets.

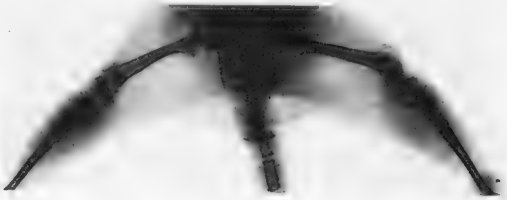
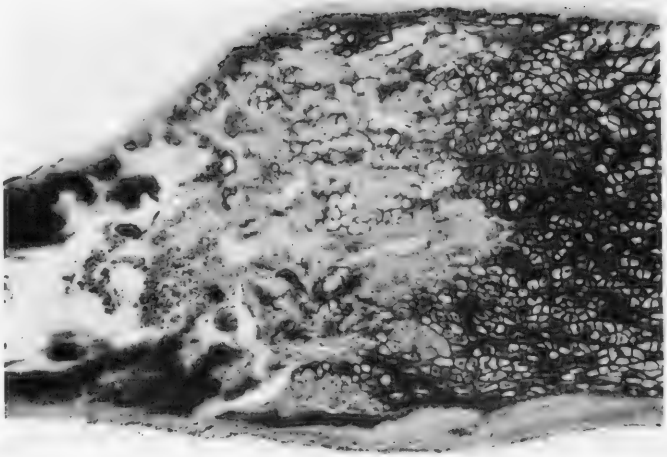


FIG. 1.



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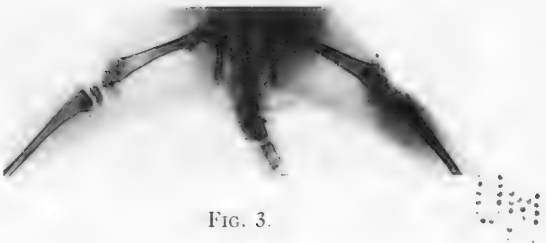
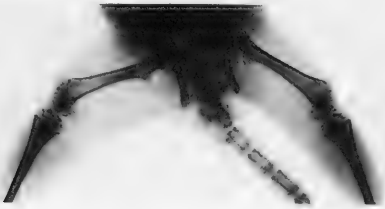


FIG. 3.

(Pappenheimer, McCann, and Zucker: Experimental rickets. V.)



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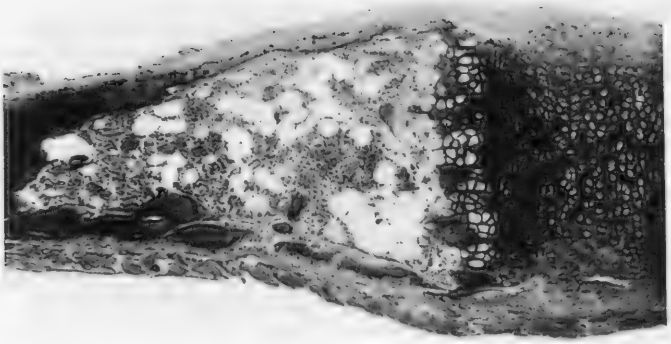

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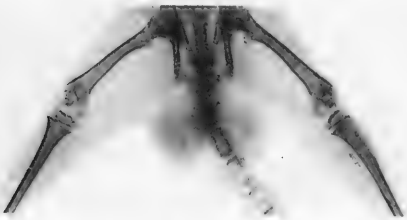


FIG. 6.

FIG. 7.

(Pappenheimer, McCann, and Zucker: Experimental rickets. V.)

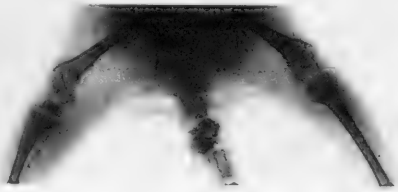
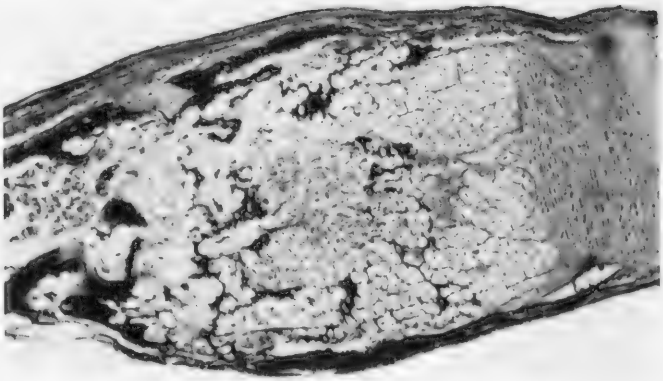


FIG. 8.



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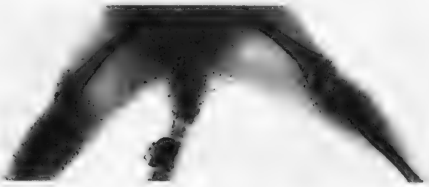

FIG. 10.

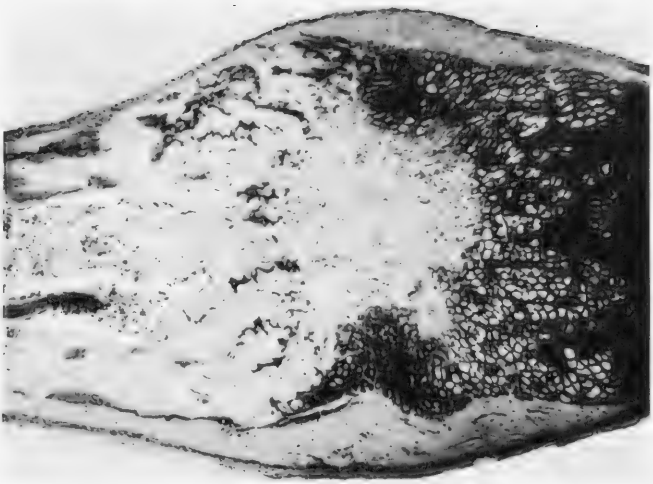

FIG. 11.

(Pappenheimer, McCann, and Zucker: Experimental rickets. V.)

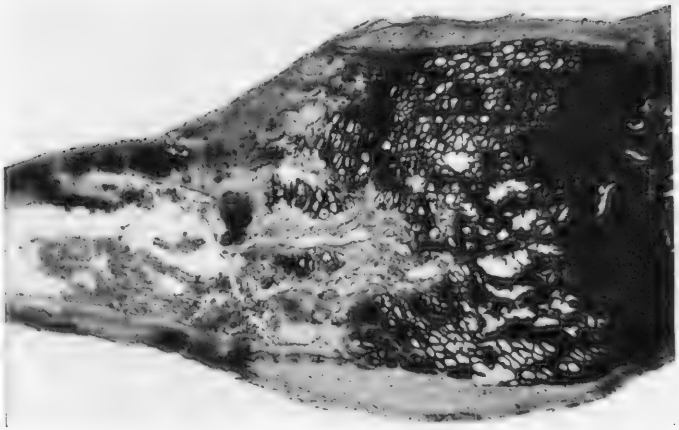

FIG. 12.

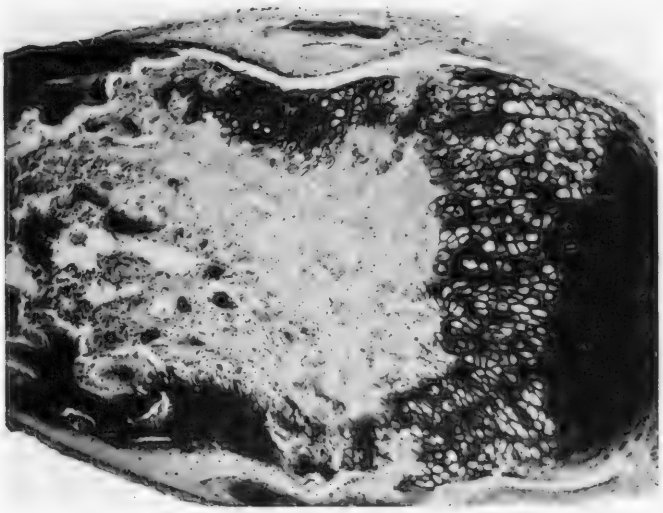

FIG. 13.

(Pappenheimer, McCann, and Zucker: Experimental rickets. V.)

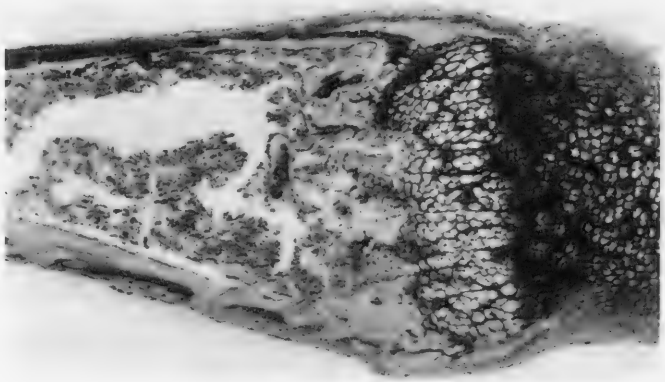

FIG. 14.

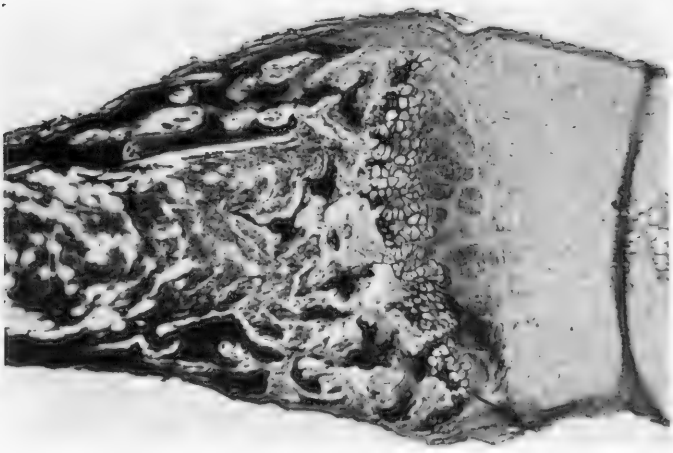

FIG. 15.

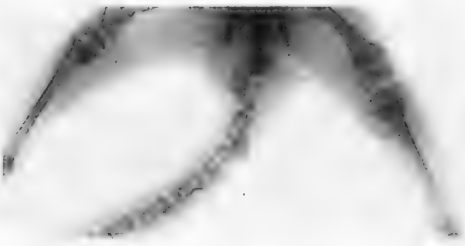

FIG. 16.

(Pappenheimer, McCann, and Zucker: Experimental rickets. V.)

MODIFICATION OF AN IMPROVED ANAEROBE JAR.

By J. HOWARD BROWN, Ph.D.

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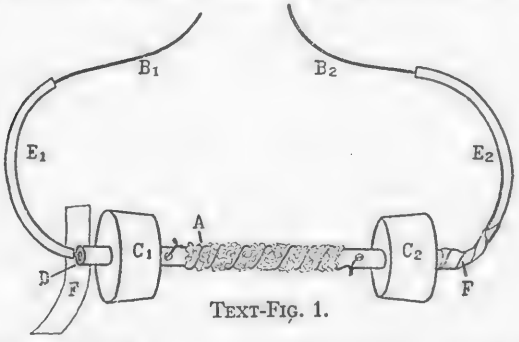
(Received for publication, November 17, 1921.)

The explosion of one of our anaerobe jars after use for a year without accident has led to a slight modification of the apparatus previously described.[1] Reference to Text-figs. 1 and 2 of the article referred to will show the copper wire terminals B_1 and B_2 leading through the rubber stoppers C_1 and C_2 beside the glass tubing D. It has been found that after some months of use the copper wires become corroded where they are in contact with the rubber stoppers, probably due to the interaction of moisture, heat, and some constituent of the rubber stoppers (possibly sulfur). The accident referred to was due to the breaking of one of these corroded wires resulting in the production of a spark just outside the rubber stopper when the electric current was turned on. The corrosion may be avoided by having the copper wires enter the coil through the bore of the glass tube D, joining the nichrome wire A through small holes in the side of the tube within the coil. Although the capillary rubber tubing E_1 and E_2 used for insulation has been found to have no appreciable corrosive action on the copper wires it is further suggested that for B_1 and B_2 one may



Text-Fig. 1.

use wire insulated with a non-corrosive insulation such as asbestos, thus eliminating the rubber tubing altogether. We also pack the bore of the glass tube with asbestos at either end where the wires pass into it and wrap the joint with insulating tape (F). The modifications recommended are shown in Text-fig. 1.

[1]Brown, J. H., An improved anaerobe jar, *J. Exp. Med.*, 1921, xxxiii, 677.

EXPERIMENTAL STUDIES ON THE ETIOLOGY OF TYPHUS FEVER.

IV. Immunizing and Toxic Agents Found Occasionally in Filtrates of Typhus-Infected Tissues.

By PETER K. OLITSKY, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 30 and 31.

(Received for publication, November 7, 1921.)

In a previous article[1] we stated that typhus virus present in tissues of guinea pigs reacting to the experimental disease failed to pass through Berkefeld V and N candles. Reference was also made to the non-filterability of the virus in the blood of infected animals.

The object of this paper is to show that these filtrates, which are free, so far as we can ascertain, from a living, multiplying agent, can occasionally induce in guinea pigs not only the typical lesions of the disease but also immunity to later injections of the active virus.

Method.

The brain and spleen obtained from guinea pigs during the height of experimental infection were chosen as sources of typhus virus, because these organs contain the virus in greater concentration than the blood.[2,3]

The tissues were disintegrated, thus liberating the virus, and were suspended in Ringer's solution. The method has been described in the previous paper.[1] 1 cc. of the unfiltered suspension was injected intraperitoneally in guinea pigs, which served as controls in demonstrating the activity of the infected tissues. Other portions of the suspensions were passed through tested Berkefeld filters (V and N) and 5 to 10 cc. were similarly injected into thirty-nine test animals. The subsequent disposition of these animals was as follows:

(*A*) Nine were bled between the 4th and 10th days after inoculation by cardiac puncture and 3 cc. of the defibrinated blood were injected intraperitoneally

[1] Olitsky, P. K., *J. Exp. Med.*, 1922, xxxv, 121.

[2] Landsteiner, K., and Hausmann, W., *Med. Klin.*, 1918, xiv, 515.

[3] Weil, E., Breinl, F., and Gruschka, T., *Wien. klin. Woch.*, 1921, xxxiv, 459.

into each of eighteen normal animals. This was for the purpose of disclosing any living microorganism in the filtrates.

(*B*) Twenty were observed for periods up to 16 days for clinical signs, especially early or late febrile reactions.

(*C*) Nine of Group B were reinjected with active blood from 10 to 16 days after the first inoculation. This was done to determine the immunizing power of filtered virus.

(*D*) Finally, ten were killed from 4 to 7 days after inoculation in order to note any changes produced in the tissues.

RESULTS.

The following is a summary of the results of the experiments.

Activity of the Virus Employed.—All the guinea pigs which were injected with the suspensions of unfiltered typhus-infected tissue showed, after an average incubation period of 7 to 8 days, a febrile reaction lasting, as a rule, for 8 days. After recovery, the animals remained resistant to later injections of active blood. Those killed at the height of reaction exhibited the characteristic histopathological changes of experimental typhus fever. With the blood obtained in this stage, a disease of similar type was produced in normal animals. No secondary or concurrent infections were found. The disease was, therefore, typical of experimental typhus fever in guinea pigs, as described in detail in another communication.[4]

Absence of Living Organisms.—The blood of guinea pigs which were inoculated with filtrates failed in each instance to induce in normal animals the typical fever observed in the controls. It must be concluded that the filtrates, in view of the failure to reproduce the disease by transmission, were free from a living, multiplying agent.[5]

Temperature Reactions.—Of the twenty guinea pigs inoculated with filtrates, fifteen revealed no disturbance of temperature. Of the remaining five, all showed a rise in temperature, above 40°C. (104°F.), for 1 to 3 days, first noted from 2 to 5 days after the inoculation.

Immunity.—Of the nine guinea pigs inoculated with filtrates and reinjected after 10 to 16 days with active typhus virus, six reacted typically to the second injection, and two showed a mild fever, lasting

[4] Olitsky, P. K., *J. Exp. Med.*, 1921, xxxiv, 365.

[5] Experiments demonstrating the non-filterability of the typhus virus have been described in the previous paper.[1]

for 4 days, instead of 8 days as in the controls, which on transmission proved to be the familiar experimental typhus, while one was not affected. Thus, the filtrates induced no immunity in six, a partial immunity in two, and a complete immunity in one of the animals.

Production of Lesions.—In four of ten guinea pigs inoculated with filtrates and killed 7 days after injection, the following lesions were found.

Macroscopic Examination.—The organs were normal in appearance, with the exception of the spleen, which was slightly enlarged and darker than is usual. In two guinea pigs a few small discolored areas, resembling the spots of a petechial rash, were seen after stripping the corium of the skin.

Microscopic Examination.—The central nervous system showed no meningeal involvement, except in its blood vessels. Focal accumulations of macrophages, or mononuclear cells, were noted about the capillaries and arterioles, which at some points were sufficient to form a distinct nodular mass giving rise to a periarteriolitis nodosa (Fig. 1). The endothelium of the vessels, often swollen, was necrotic in some portions and proliferated in others. Occasionally an arteriole or capillary was noted to contain occluding thrombi. The gray matter of the brain, especially that of the midbrain, contained a few small localized hemorrhages. The vessels here revealed lesions similar to those found in the vessels of the meninges. Nodules were seen, always in proximity to the vessels, which consisted mainly of macrophages, or mononuclear cells, and a few polymorphonuclear cells (Fig. 2).

The heart tissue revealed no visible changes except for a few localized hemorrhages and the vascular lesions described above (Figs. 3 and 4).

The changes in the spleen were such as to obscure the vascular lesions. There were noteworthy congestion and leucocytic infiltration. The lymphoid follicles were enlarged and at the center were a number of macrophages replacing to some extent the lymphoid cells.

The skin in two of the guinea pigs exhibited in the deeper layers of the corium small localized hemorrhages and the vascular lesions and nodules described above (Fig. 5).

The lesions found in these animals are characteristic of experimental typhus fever in the guinea pig.[4]

To summarize the results, it appears that in a small number of instances other effects than typhus are produced by the inoculation of filtrates from typhus-infected tissues. In five of twenty guinea pigs, a rise in temperature occurred which lasted from 1 to 3 days. One of nine guinea pigs remained immune and two others of this

series responded with a mild reaction, after a test injection with active virus. Finally, four of ten animals showed the characteristic lesions of the experimental disease.

DISCUSSION.

We have already demonstrated[1] that the typhus virus present in the tissues of guinea pigs at the height of their reaction to inoculation is not filterable through tested Berkefeld filters. Hence effects produced by means of filtrates of infected tissues cannot be attributed to a living, multiplying agent. The possibility of the presence of subinfective quantities of the virus in filtrates producing the results can be eliminated by the fact that whereas only infinitesimal amounts of infected brain tissue (0.005 gm.)[2] are necessary to induce the experimental infection, we have employed almost one-half of the amount of entire filtrate obtained from suspensions of all the brain tissue from 300 to 350 gm. guinea pigs. Furthermore, in our experience[1] transmission of the virus from animal to animal by means of filtrates has failed.

The experiments reported in this paper demonstrate that such filtrates can produce occasionally an early and short febrile reaction, immunity to later injections of active virus, and lesions indistinguishable from those of the typical experimental disease. From this we infer that a specific substance may be present in the tissues of infected guinea pigs and be occasionally obtainable in filtrates.

Nicolle and his associates[6] have maintained that the typhus virus is filterable for the reason that infrequently monkeys which have been inoculated with filtrates of blood, or of lice harboring the virus, have shown no definite temperature reaction, but remained resistant to a later injection of active virus. The experiments above described demonstrate that such occasional immunity following inoculations of filtrates depends on some other factor than a living, multiplying agent.

[6] Nicolle, C., Conor, A., and Conseil, E., *Compt. rend. Acad.*, 1910, cli, 685; 1911, cliii, 578. Nicolle, C., Blanc, G., and Conseil, E., *Arch. Inst. Pasteur Tunis*, 1914, ix, 84.

CONCLUSION.

In the filtrates of typhus-infected tissues of guinea pigs can be occasionally found a substance which produces in these animals thermic reactions, lesions characteristic of experimental typhus, and, still less frequently, immunity to later injections of active virus. The general indications are that this substance is not a living organism.

EXPLANATION OF PLATES.

PLATE 30.

FIG. 1. Section of the brain of a guinea pig inoculated with a filtrate of typhus-infected tissue. The vessels of the meninges show the perivascular accumulation of macrophages of the nature of a nodular formation and occlusion by thrombus of other vessels. A small nodule can be seen in the gray matter near the surface. × 240.

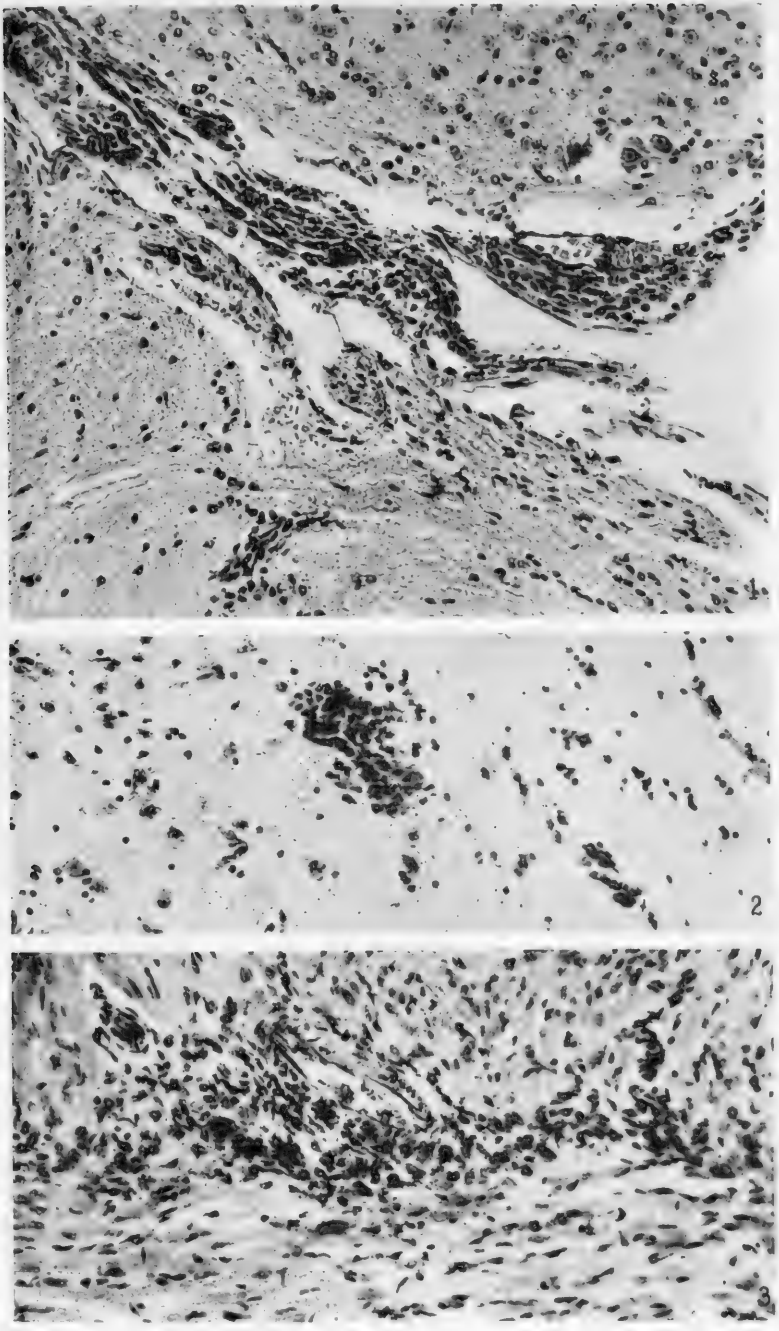
FIG. 2. Another section of the same brain. A distinct nodule in the gray matter can be observed. × 240.

FIG. 3. Section of heart tissue from the same guinea pig, showing vascular lesions similar to those in the brain. × 240.

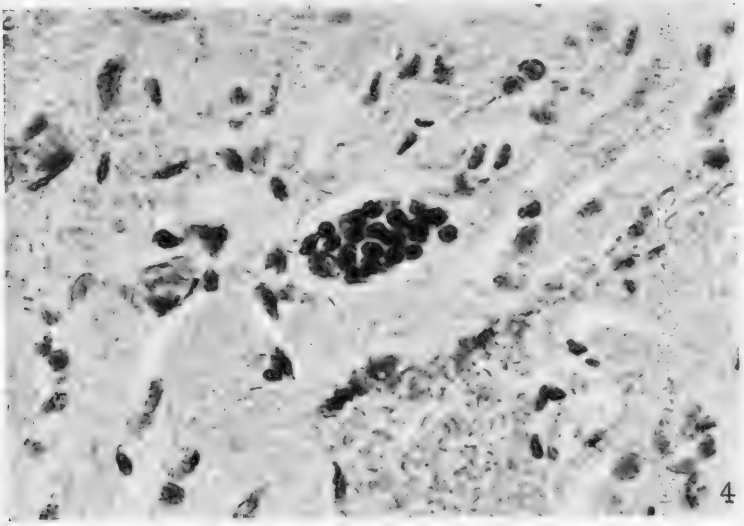
PLATE 31.

FIG. 4. Another section of heart tissue. A nodule under higher magnification to show its structure. × 560.

FIG. 5. Section of the deeper layers of the skin from the same guinea pig. At the edge of the section, toward the epithelial surface, is a small nodule indicated by an arrow; in the deeper muscular layer is a pronounced vascular lesion similar to that noted in Fig. 1. × 240.



(Olitsky: Etiology of typhus fever. (V.)



(Olitsky: Etiology of typhus fever. IV.)

STUDIES ON X-RAY EFFECTS.

X. The Biological Action of Small Doses of Low Frequency X-Rays.

By WARO NAKAHARA, Ph.D., and JAMES B. MURPHY, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 32 and 33.

(Received for publication, November 2, 1921.)

Attempts have been made in this laboratory to compare the biological action of the soft or low frequency and the hard or high frequency x-rays, but the impossibility of comparing doses of the two types of rays with our present apparatus and measuring devices has rendered the results of these studies of little value. However, by varying the doses of the soft and hard rays there is one point which seems definite; namely, that with the softer rays it is possible to induce an apparent stimulation of the lymphoid cells which is preceded by only a very short and transitory period of depression.[1–4] With the harder rays the stimulation phase is less pronounced, if present at all in the dosages employed by us. Russ, Chambers, Scott, and Mottram[5] have succeeded in bringing about an increase in the circulating lymphocytes of rats by repeated small exposures to x-rays described by them as being of "medium" or "medium soft" quality. Our first satisfactory results were obtained with the tube operated at a spark-gap of about $1\frac{1}{2}$ inches. Later experiments with a spark-gap of about $\frac{7}{8}$ inch gave a more uniform and pronounced reaction.

The present study has to do with the effect of still softer x-rays than those used in previous experiments. For the generation of these

[1] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 800.

[2] Thomas, M. M., Taylor, H. D., and Witherbee, W. D., *J. Exp. Med.*, 1919, xxix, 75.

[3] Nakahara, W., *J. Exp. Med.*, 1919, xxix, 83.

[4] Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1920, xxxi, 13.

[5] Russ, S., Chambers, H., Scott, G. M., and Mottram, J. C., *Lancet*, 1919, i, 692.

rays a special water-cooled tube has been devised with a window of thin glass which will allow the passage of rays usually held back by the thicker glass of the standard tubes.[6]

Production of Lymphocytosis.

The following experiments were planned to determine the time of exposure to the soft rays necessary for the production of a maximum stimulation of the lymphoid system of mice.

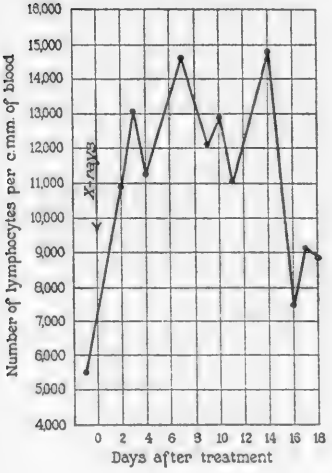
Experiment 1.—Forty-five white mice were divided into nine groups of five mice each. The groups were exposed to x-rays for ½, 1, 2½, 4, 5, 10, 20, 30, or 60 minutes respectively. The special tube described above was used with ½ inch spark-gap, 11 milliamperes, and 6 inches distance, for the treatment. A cardboard was placed over the animal container for protection against the heat. Blood counts were made on all of the animals before and 1 week after the treatment. The mice given the 1 minute exposure showed a definite lymphocytosis, but no consistent change was noted in the blood picture of the other groups.

Experiment 2.—Blood counts were made on thirty normal white mice and they were then exposed to x-rays given by the special tube with the governing factors the same as in Experiment 1, the length of the exposure being 1 minute. Counts were made at intervals afterwards. The changes in the lymphocytes are shown in Text-fig. 1 by a composite curve and the results for the individual animals in Table I.

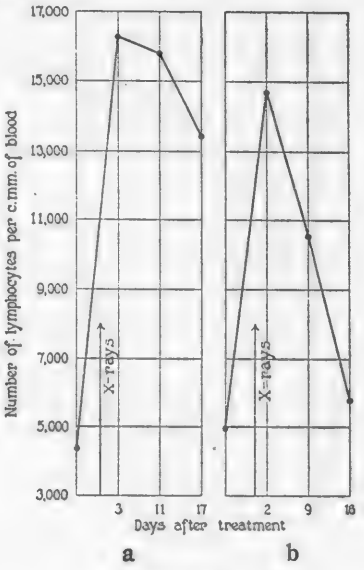
The counts showed that there was no definite alteration in the absolute number of polymorphonuclear leucocytes, but the lymphocytes were increased to a level considerably above that of the initial count. By the 2nd day these latter cells had almost doubled their number and continued to increase with some variations until the 14th day, after which they subsided but were still above their initial level on the 18th day. There was considerable variation in the extent and duration of the reaction in individual mice, as shown in Table I and in Text-fig. 2. In only two animals was there a drop in the lymphocytes at the time of the first count after the x-ray treatment. One of these, however, showed a reaction later with an increase of over 30 per cent, but in the other no stimulation was observed. It should be noted here that none of these counts were made soon enough after the

[6] We wish to acknowledge our indebtedness to the Research Laboratory of the General Electric Company for the design and construction of this tube.

x-ray treatment to demonstrate the amount of depression preceding the stimulation, for this point has been adequately dealt with in previous publications.[7] The increase in number of circulating lymphocytes after this dose of x-rays is of greater magnitude and more constant than that previously induced by this agent.



TEXT-FIG. 1.



TEXT-FIG. 2.

TEXT-FIG. 1. Composite curve of lymphocyte counts on thirty-five mice before and after an exposure to low frequency x-rays.

TEXT-FIG. 2, *a* and *b*. (*a*) Curve of lymphocyte counts on Mouse 23 in Table I. (*b*) Curve of lymphocyte counts on Mouse 12 in the same table.

[7] In a recent article Mottram and Russ (Mottram, J. C., and Russ, S., *J. Exp. Med.*, 1921, xxxiv, 271) have objected to our use of the term "stimulating" doses of x-rays in describing the effect of this agent on the lymphoid tissue. They object to this term on the basis of the fact that the stimulation is preceded by a short period of depression. This point was noted in our first publication on the subject[1] and has been repeatedly referred to in subsequent articles.[4,9] The fact that with a suitable small dose of x-rays a stimulation of the lymphoid tissue is produced with only a slight and transitory depression, while with larger doses a more marked and lasting depression is induced with no subsequent stimulation, seems to us to justify the use of the expression.

TABLE I.

Mouse No.	Count before treatment.	Count after treatment. No. of days after x-ray treatment. 2	3	4	7	9	10	11	14	16	17	18
1	6,018				15,908				13,250			
2	6,541				11,105				17,713			8,430
3	5,897				14,455							
4	5,899				16,210				14,446			
5	5,526				15,463				14,744			
6	5,823	11,041				12,252				7,091		
7	4,617	7,658										
8	4,336	12,070				16,532				11,645		
9	6,585	17,070				10,603				8,455		
10	4,239	10,972				13,650				9,423		
11	4,288	7,604				11,766				5,471		
12	4,998	14,734				10,567				5,816		
13	7,104	7,070				12,786				7,005		
14	5,477	7,406				10,472				6,808		
15	5,185	12,878				10,332				6,520		
16	6,110		16,122				12,800				8,888	
17	6,113		12,775				16,861				7,1 3	
18	4,215		9,435				12,890				6,008	
19	4,909		12,002				7,338				5,360	
20	4,790		7,007				9,495				9,231	
21	3,714		12,064				9,304				8,232	
22	4,145		10,989				12,543				9,382	
23	4,425		16,261				15,814				13,209	

24	6,203		18,252				15,681				12,124	
25	7,155		15,352				17,055				12,836	
26	3,594			7,527				9,303				
27	9,313			7,027				8,975				9,897
28	4,428			8,815				7,196				6,675
29	5,264			3,767				7,986				6,878
30	4,404			12,452				12,870				10,178
31	3,974			15,300				15,082				
32	8,330			13,167				10,277				8,571
33	7,329			16,480				17,274				14,796
34	6,175			16,787				12,894				9,893
35	5,635			11,851				8,518				5,092
Average.....	5,507	10,850	13,026	11,317	14,628	12,105	12,978	11,037	15,038	7,581	9,245	8,934

The counts before treatment were made a number of days in advance so that no animal had more than one count in a week. For example, on the group of mice counted 2 days after treatment, the pretreatment count was made 5 days before, thus allowing a week between counts.

Histological Changes.

The material for this study was collected from twenty-six mice which were carried as a parallel series to the above experiment in which blood changes were observed. After exposure to x-rays (special tube, spark-gap $\frac{1}{2}$ inch, milliamperes 11, distance 6 inches, time 1 minute) these animals were killed in groups at intervals of 24 hours, 4 days, 7 days, 10 days, and 14 days. The spleen, lymph nodes, bone marrow, thymus, thyroid, liver, kidney, suprarenal, ovary, and testis were taken for examination. Carnoy 6–3–1 solution was used for fixation and the sections were stained with Heidenhain's iron-hematoxylin for mitotic figures or with Ehrlich's acid hematoxylin and eosin.

Lymphoid Organs.—The changes in the spleen and lymph nodes were practically identical and will be described together. 24 hours after treatment there was no demonstrable change in the lymphoid organs. At the 4 day period numerous mitotic figures were found in all of these organs, a condition which our previous work indicates is associated with the increased production of lymphoid cells. This condition was found equally as pronounced in the animals killed on the 7th day after treatment. The organs from eleven mice were examined for these two periods and without exception this increase in proliferation was observed. The animals killed on the 10th and 14th days after treatment showed that the proliferative activity had subsided to about the normal rate.

Suprarenal Glands.—The suprarenals were found at autopsy to be distinctly reddish in color and somewhat enlarged. Microscopic examination showed the sinus-like spaces between the cortex and medulla to be much distended with blood, and this dilatation extended to the capillaries between the cortical cell columns, separating these columns by a wide margin (Figs. 1 and 2). The sinusoids of the medulla showed little if any modification. There was no sign of hemorrhage or of necrosis of the suprarenal tissue in any of the specimens examined. This vascular change was observed in varying degrees in all of the mice examined except those killed 24 hours after the treatment. In addition to this engorgement of the capillaries, a proportion of the animals killed between 7 and 14 days after the

x-ray treatment showed a pronounced perivascular infiltration in the cortex along the fibrous capsule and often extending down to the zona reticularis along the cortical cell columns.

Other Organs.—There was no histological evidence of changes in the other organs examined after the x-ray treatment. Even the germ cells of the fourteen ovaries and twelve testes examined, cells well known to be extremely sensitive, showed no damage which could be detected microscopically (Figs. 3 and 4). In a few instances the interstitial tissue of the testis was found to be hypertrophied. Distinct perivascular lymphoid infiltration was observed in a number of the livers and kidneys examined, but these findings were not uniform enough to be of great importance. This condition is also found occasionally in normal animals.

Effect on Resistance to Transplantable Cancer.

In previous publications it has been shown that a small dose of x-rays sufficient to stimulate the lymphocytes will increase the resistance of mice to transplanted cancer.[1,8,9,10] Russ, Chambers, Scott, and Mottram[5] have shown that the same condition holds for rats. As noted above, a more pronounced reaction of the lymphoid tissue is induced by the treatment outlined here than by the dosage previously used. It is of interest, therefore, to determine the effect of the very soft rays on the resistance to cancer.

Experiments 3 to 9.—Normal white mice of about the same age and size were exposed to a dose of x-rays governed by the same factors as those used in Experiment 2, with the special x-ray tube described above. These animals were inoculated in groups from 3 to 14 days after the treatment, with a graft of a transplantable tumor (Bashford Adenocarcinoma No. 63) along with a suitable number of controls. The results of seven such experiments are given in Table II.

From these experiments it is seen that no immunity is evident when the inoculation is made as early as 3 days after the x-ray treatment, a result which corresponds with the earlier experiments in which the standard Coolidge tube was used. Among 86 mice inoculated from 7

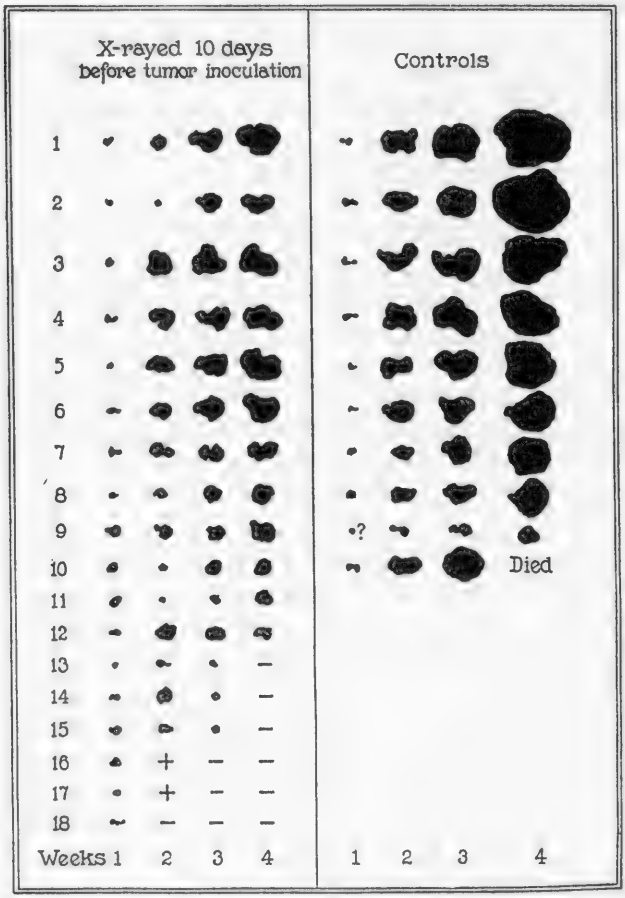
[8] Murphy, Jas. B., *Proc. Nat. Acad. Sc.*, 1920, vi, 35.

[9] Murphy, Jas. B., Nakahara, W., and Sturm, E., *J. Exp. Med.*, 1921, xxxiii, 423.

[10] Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1921, xxxiii, 429.

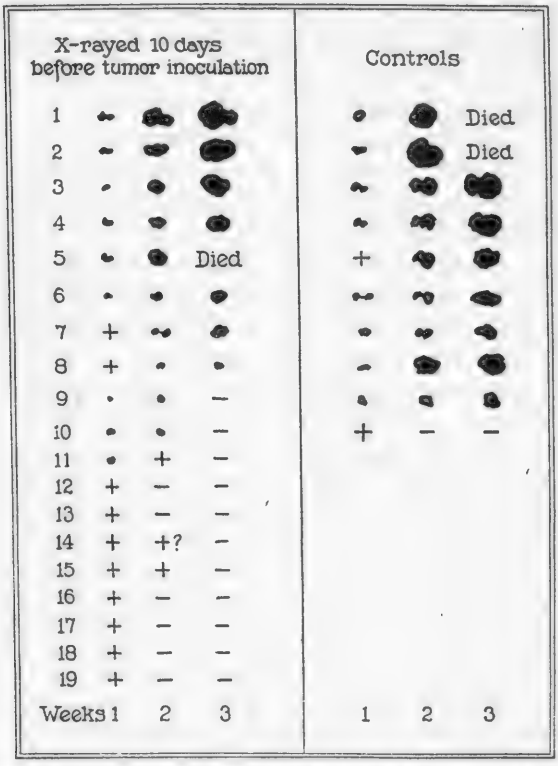
TABLE II.

Experiment No.	Interval between x-ray exposure and tumor inoculation.	Immunity in x-rayed animals.	Immunity in control animals.
	days		
3	3	0.0 per cent (9 mice).	0.0 per cent (10 mice).
4	7	30.0 " " (10 ").	11.1 " " (9 ").
5	7	28.5 " " (17 ").	9.9 " " (11 ").
6	10	37.5 " " (8 ").	0.0 " " (10 ").
7	10	33.3 " " (18 ").	0.0 " " (10 ").
8	10	57.8 " " (19 ").	10.0 " " (10 ").
9	14	20.0 " " (10 ").	0.0 " " (7 ").



TEXT-FIG. 3. Experiment 7. The rate of growth of Bashford Adenocarcinoma No. 63 in mice given a small dose of low frequency x-rays 10 days before inoculation, contrasted with the rate of growth in untreated mice.

to 14 days after the treatment, 34.4 per cent were immune, while in 57 control mice inoculated with the same tumor there were only 5.1 per cent immune. The largest proportion of resistant mice was found among the animals treated 10 days after inoculation, with 42.8 per



TEXT-FIG. 4. Experiment 8. The rate of growth of Bashford Adenocarcinoma No. 63 in mice given a small dose of low frequency x-rays 10 days before inoculation, contrasted with the rate of growth in untreated mice.

cent in 45 mice against 3.3 per cent in 30 control mice. Text-figs. 3 and 4 show that the rate of growth of the tumor is slower in the treated animals than in the controls, and that in a proportion of the treated mice tumors after a period of growth are absorbed, a condition which rarely occurs with this tumor in normal mice.

The Absorption Coefficient of X-Rays Produced by a Voltage Measured by a ½ Inch Spark-Gap.[11]

It is of considerable interest in the light of the above experiments to arrive at some idea of the amount of penetration of the soft rays. The very soft rays produced by a voltage measured by a ½ inch point spark-gap were found to be practically homogeneous with a mass absorption coefficient equal to 3.4 for water. This value includes the mass-scattering coefficient usually taken as 0.2.

The value was measured photographically. A series of small areas of a single film was exposed to the rays for various times. A second series on the same film was exposed for various times to rays which had passed through layers of water of various thicknesses. After development the areas of the second series were matched for blackness against those of the first series and the relative intensities of the rays transmitted by the water thus discovered. A preliminary experiment gave the value for the absorption coefficient which was used to calculate the exposures through various depths of water necessary to give equal degrees of blackening. Another film was made accordingly and the exposed areas were found to be of uniform density. It will be noticed that, according to the quantum law, the wave-lengths produced by a ½ inch gap are in the neighborhood of the K characteristic absorption discontinuities of the silver and bromine of the plate. In spite of this it can be shown that the constant value found for the absorption coefficient is a proof of homogeneity.

The mass absorption coefficient of these rays in animal tissues may be assumed to be somewhat smaller than for water. A mouse skin weighing 0.2 gm. per cm. should stop less than one-half of the rays.

The relative amount penetrating various depths of water when this water-cooled tube is operated at ½ inch spark-gap is as follows:

Depth.	Intensity.
cm.	
0	1.0 (taken as standard).
¼	0.43
½	0.18
¾	0.076
1	0.032
1¼	0.0133
1⅓	0.0056

[11] We are indebted to Dr. Harry Clark for the measurements recorded.

DISCUSSION.

The comparison of the biological effect of the hard and soft rays is a matter of considerable interest at the present moment but no really satisfactory standard of measurement is available. Such comparisons, therefore, must await a further understanding of the complex nature of the biological changes and the underlying factors bringing about these changes.

As far as our present knowledge extends the only known physical or chemical change induced by x-rays depends on the power of this agent to ionize. If ionization be the underlying factor responsible for the biological changes, it is necessary to determine why some types of animal cells are so profoundly affected in the absence of demonstrable changes in other cells. However, the solution of such problems as this must await further development in biophysics.

In this communication evidence is presented of a biological change induced by a small dose of the very soft x-rays; namely, a stimulation of the lymphoid cells preceded in all probability by a small amount of destruction. We have not been able to induce a reaction of this nature with the harder rays given in varying dosage. Until it is determined that the dosage is the same it will not be possible to say whether this apparent difference in biological action of the soft and hard rays is a real one.

Considering the smallness of the dose, the fact that 57 per cent of the x-rays used here is absorbed in the first $\frac{1}{4}$ cm. of tissue and that only 3.2 per cent penetrated to the depth of 1 cm. strongly suggests that the changes in the lymphoid organs are not the result of the direct action of the x-rays. The fact also that the deeper nodes react as much as the more superficial ones strengthens this idea. The significance of the changes in the suprarenals can only be determined by further study. It is noteworthy that the testicle and ovary, organs supposed to be extremely sensitive, were unaffected by this treatment.

The virulence of the strain of tumor used to test the resistance of the mice was such that the so called natural resistance was almost completely obviated, a fact which renders the result of the inoculation of the treated mice the more striking.

SUMMARY.

A study has been made of the biological effect of a small dose of soft x-rays given off by a special water-cooled tube with a window of thin glass, operated at ½ inch spark-gap and 11 milliamperes. Mice exposed for 1 minute show 2 days later in the blood an increase in the number of lymphocytes and in the lymphoid organs an increased number of mitotic figures. There occurs also a marked dilatation of the vessels of the suprarenals, particularly between the cortex and medulla. The latter condition did not appear until after 24 hours and was still present 14 days after the treatment. No change was detected in other organs.

Mice treated in this way showed a high degree of resistance to cancer transplants. The amount of resistance varied with the time of the inoculation after the treatment. The resistance was not increased before 3 days after and was at its highest point 10 days after the treatment.

EXPLANATION OF PLATES.

PLATE 32.

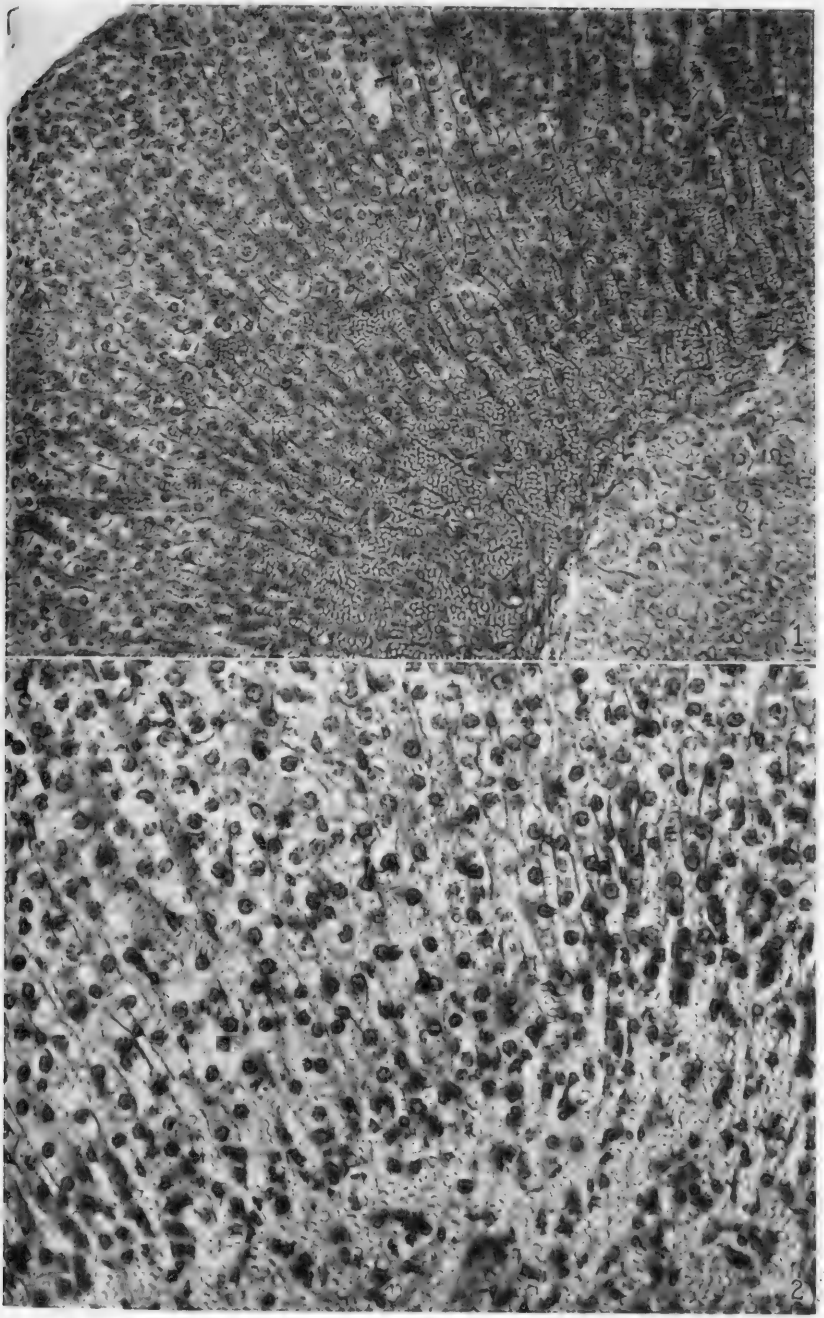
FIG. 1. Suprarenal gland of mouse 7 days after an exposure to low frequency x-rays, showing dilatation and engorgement of capillaries.

FIG. 2. The same as Fig. 1; higher power view.

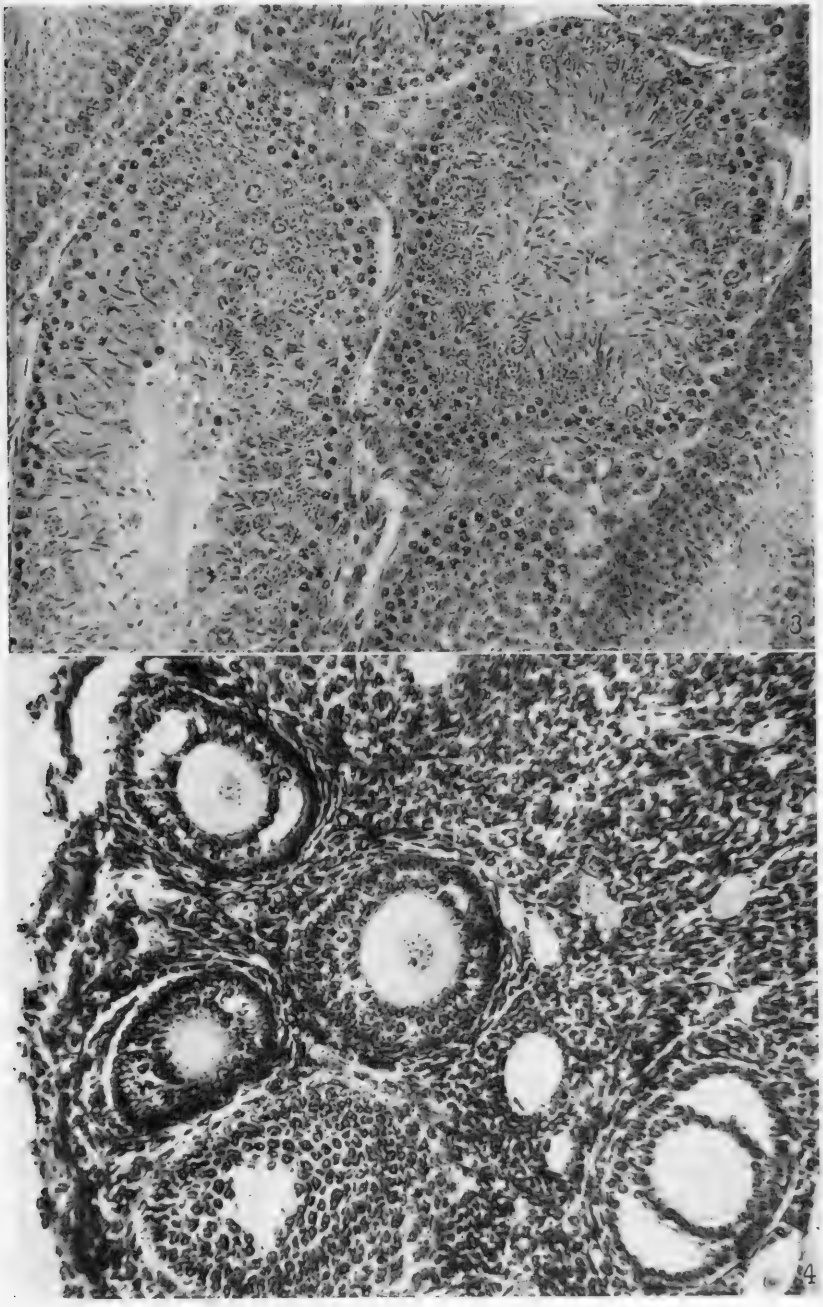
PLATE 33.

FIG. 3. Testis of mouse 24 hours after an exposure to low frequency x-rays. Spermatocytes in the periphery of follicles in the so called syncopic stage. In the large follicle to the left are seen several examples of the first spermatocytic division.

FIG. 4. Ovary of mouse 24 hours after an exposure to low frequency x-rays.



(Nakahara and Murphy: Studies on x-ray effects. X.)



(Nakahara and Murphy: Studies on x-ray effects. X.)

STUDIES ON X-RAY EFFECTS.

XI. The Fate of Cancer Grafts Implanted in Subcutaneous Tissue Previously Exposed to X-Rays.

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(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, November 22, 1921.)

It has been shown in this laboratory[1] that an erythema dose of x-rays produces in the skin layers of mice a reaction characterized by lymphoid infiltration coincident with a local increase in resistance to transplanted cancer. Moreover, it was found that while the x-rayed areas are refractory to subsequent intracutaneous inoculation of cancer, subcutaneous inoculations beneath the x-rayed areas result in the same number of growths as in the normal areas. These experiments were offered as a probable explanation of the fact that many skin cancers in man are readily influenced by x-rays while identical cancers in the superficial lymph nodes are controlled with great difficulty, if at all, by the treatment.

The exposure of open wounds to x-rays at the time of operation for the removal of cancer in man has been frequently advocated, particularly in cancer of the breast[2] for the purpose of destroying any remaining cancerous tissue. While the results are stated to have been satisfactory, it is difficult to judge the value of the method in the treatment of human cancer in which it is impossible to provide a suitable number of controls.

Hill, Morton, and Witherbee have shown that mouse cancer cells are not killed by x-rays *in vitro* in doses much greater than those tolerated by the skin.[3] Yet as stated above, a much smaller dose will render the skin resistant to implants of a strain of this same cancer.

[1] Murphy, Jas. B., Hussey, R. G., Nakahara, W., and Sturm, E., *J. Exp. Med.*, 1921, xxxiii, 299.

[2] Pfahler, G. E., in Deaver and McFarland, The breast, its anomalies, its diseases and their treatment, Philadelphia, 1917, 651.

[3] Hill, E., Morton, J. J., and Witherbee, W. D., *J. Exp. Med.*, 1919, xxix, 89.

Hence it became desirable to determine whether or not direct exposure of the deeper tissues renders them refractory to implanted cancer.

Tumor Inoculation after Exposure of the Subcutaneous Tissue to X-Rays.

Normal white mice were etherized, shaved over the abdomen, and under aseptic conditions a rectangular skin flap was made beginning at the midline and extending about 1.5 cm. laterally across the abdomen. The incision was made so as to leave the skin attached at the distal end and the flap was separated from the underlying structures so as to include all of the subcutaneous tissue down to the muscle. The under side of the flap and the exposed muscle, after being covered with gauze wet with salt solution, were given directly a dose of x-rays governed by the following factors: 3 inch spark-gap, 10 milliamperes, 6 inch distance, $2\frac{1}{2}$ minutes. With the exception of this area the animal's body was protected by sheet lead. Immediately after the treatment a cancer graft was introduced into the loose connective tissue of the under side of the flap and the skin sutured back into place. As a control, another series of animals was treated in precisely the same fashion except that no x-rays were given.

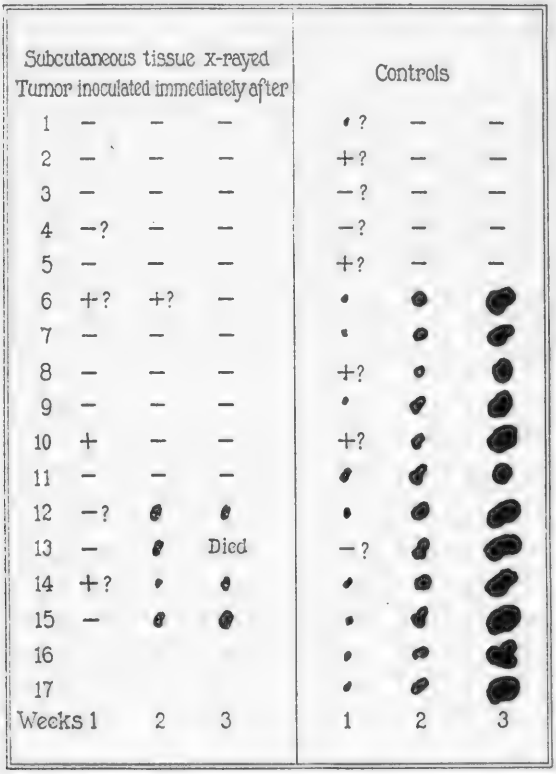
In practically all of the animals of both series the wounds healed within 5 or 6 days by primary intention, with no detectable difference between the x-rayed and control animals. Weekly examinations were made to determine the fate of the cancer grafts and later verified by autopsy.

The results of four such experiments are given in Table I.

TABLE I.

Experiment No.	Resistant x-rayed mice.	Resistant control mice.
1	66.6 per cent (15 mice).	23.0 per cent (13 mice).
2	68.4 " " (19 ").	0.0 " " (6 ").
3	73.3 " " (15 ").	29.4 " " (17 ").
4	50.0 " " (10 ").	0.0 " " (10 ").
Average...	66.1 per cent (59 mice).	17.4 per cent (46 mice).

It is apparent from these experiments that an erythema dose of x-rays given directly to the subcutaneous tissue brings about some change which renders this tissue decidedly less suitable as a soil for the growth of implanted cancer (Text-fig. 1). Another point of


Subcutaneous tissue x-rayed
Tumor inoculated immediately after
Controls
1 − − − • ? − −
2 − − − + ? − −
3 − − − − ? − −
4 − ? − − − ? − −
5 − − − + ? − −
6 + ? + ? − • ● ●
7 − − − • ● ●
8 − − − + ? ● ●
9 − − − • ● ●
10 + − − + ? ● ●
11 − − − ● ● ●
12 − ? ● ● • ● ●
13 − ● Died − ? ● ●
14 + ? • ● ● ●
15 − ● ● • ● ●
16 • ● ●
17 • ● ●
Weeks 1 2 3 1 2 3

TEXT-FIG. 1. Results of inoculation of tumor into subcutaneous tissue previously exposed directly to x-rays, compared with a like inoculation in normal mice.

interest is that the cancer grafts which took in the x-rayed animals showed a tendency to grow inward toward the abdominal cavity with a flat inactive base on the side near the skin. Some of these did not produce even a slight elevation of the overlying skin and were only detected at autopsy.

To serve as a control for the above experiments and in order to confirm the finding that the effect of a local erythema dose of x-rays applied to the skin does not extend to the underlying subcutaneous tissue, the following experiment was carried out.

Subcutaneous Inoculation of Tumor after Exposure of the Skin to X-Rays.

Ten normal white mice were given a dose of x-rays over the left lower quadrant of the abdomen, the dose being governed by the same factors as those used in the preceding experiment. Immediately following the exposure, a skin flap was made in the x-rayed area and a cancer graft (Bashford No. 63) was introduced into the loose areolar tissue of the under side of the flap and the wound closed with sutures. The tumor grew in all of these animals, from which it may be concluded that an erythema dose of x-rays given to the intact skin does not increase the resistance of the underlying subcutaneous tissue.

Tumor Inoculation in a Protected Area after a Local Exposure of the Subcutaneous Tissue to X-Rays.

In order to determine whether the exposure to x-rays of a small area of subcutaneous tissue affects the general resistance to cancer grafts, a series of thirteen mice was operated upon and after the skin flap was made on the left side of the abdomen they were x-rayed over the open wound and then the flap was sutured back into place. Cancer grafts inoculated immediately afterwards in the right side resulted in tumors in 76.9 per cent of the animals, or in about the proportion observed in normal control mice.

Histological Changes after Direct Exposure of the Subcutaneous Tissue to X-Rays.

Two series of twelve mice each were shaved, and under ether flaps of skin and subcutaneous tissue were made over the left lower abdominal region. One series was x-rayed with an erythema dose directly on the under side of the skin flap and on the denuded surface of the abdominal muscle, the remaining parts of the animal being protected by sheet lead. The other series was operated on in the

same manner but not x-rayed. The wounds in both were sutured with great care as to the approximation of the skin edges. The animals were killed in groups of two, 24 hours, 3, 5, 7, 9, and 14 days after operation for examination.

Up to the 5th day the process of repair formed such a prominent part of the picture that it was impossible to detect any difference in the extent and character of the cellular infiltration from histological study. The 7 and 9 day preparations, however, in which the process of repair was in the last stages, showed distinctly that while in the animals not x-rayed only a layer of new connective tissue between the subcutaneous and muscle layers was slightly infiltrated with round cells, in the x-rayed animals large numbers of lymphocytes occurred, chiefly in the loose connective tissue, and in about half of the animals examined these cells had infiltrated the thickness of the muscle and formed a heavy layer between the muscle and the parietal peritoneum. At the end of 2 weeks this lymphocytic infiltration, although still evident, had subsided somewhat.

Two other groups of mice were operated on in the same manner as in the preceding experiment, and one of the groups was given a dose of x-rays over the exposed subcutaneous tissue and muscle. Before the skin flap was sutured back into place each animal received a cancer graft into the connective tissue underlying the flap. The microscopic appearances of the sections of tissue taken at intervals from the animals were so complicated, through operation, x-ray treatment, inoculation of tumor, natural differences in susceptibility, and in some cases, mild infections, that no conclusions in regard to the cellular reactions could be drawn.

DISCUSSION.

The observations reported in this paper bring out the fact that x-rays can be made to induce a local change in the subcutaneous tissue similar to that which this agent will induce in the skin. This change, in both instances, renders the locality resistant to the growth of implanted cancer cells, but does not affect the general resistance of the animal. This is an additional point to be taken into consideration in determining the method of treatment and the interpretation of clinical results following the use of x-rays as a therapeutic agent. The

clinician has rarely taken into account other possibilities than the direct destruction of the cancer cells.

X-rays under certain conditions materially increase the general resistance of the body to cancer, an observation made in this laboratory,[4] and later confirmed and extended by Russ, Mottram, and their coworkers.[5] On the other hand, excessive doses of x-rays are capable of lowering both natural and induced resistance to cancer.[6] The amount of this agent required to kill mouse cancer cells is many times that which can be tolerated by the skin, yet as shown above a mild erythema dose is sufficient to render the skin and under proper conditions the subcutaneous tissue antagonistic to the growth of implanted cancer. It is undetermined which of these various qualities of x-rays are responsible for the successes and failures in the treatment of human cancer. It would seem of prime importance to estimate the relative value of these effects, for it is not beyond the possible that a method of treatment could be devised which would make use of the favorable and eliminate the unfavorable action of this agent.

SUMMARY.

An erythema dose of x-rays given direct to the exposed subcutaneous tissue and muscle greatly diminishes the susceptibility of the exposed area to transplanted cancer. The same dose given over the intact skin does not affect the resisting power of the underlying subcutaneous tissue.

Histological examination shows that a few days after the exposure of the subcutaneous tissue there is a lymphoid infiltration of this tissue, which infiltration sometimes includes the muscle layers as well.

[4] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 800. Murphy, Jas. B., *Proc. Nat. Acad. Sc.*, 1920, vi, 35. Murphy, Jas. B., Nakahara, W., and Sturm, E., *J. Exp. Med.*, 1921, xxxiii, 423. Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1921, xxxiii, 429.

[5] Russ, S., Chambers, H., Scott, G. M., and Mottram, J. C., *Lancet*, 1919, i, 692. Russ, S., Chambers, H., and Scott, G. M., *Proc. Roy. Soc. London, Series B*, 1921, xcii, 125.

[6] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 204. Murphy, Jas. B., and Taylor, H. D., *J. Exp. Med.*, 1918, xxviii, 1. Mottram, J. C., and Russ, S., *Proc. Roy. Soc. London, Series B*, 1917, xc, 1.

STUDIES ON LYMPHOID ACTIVITY.

VI. Immunity to Transplanted Cancer Induced by Injection of Olive Oil.

By WARO NAKAHARA, Ph.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 34 to 36.

(Received for publication, October 31, 1921.)

It has been frequently suggested that the lymphoid cells are in some way concerned with the absorption and digestion of fats and lipoids. Recently a number of investigators have reported on the cellular changes following injections of these substances. Ramond[1] found that olive oil injected intraperitoneally is gradually absorbed by white cells of the lymphoid variety, and Clark[2] noted that the subcutaneous injection of olive oil exerts what he considers to be a chemotactic influence on the lymphatic endothelium and lymphocytes. Bergel[3] confirmed and extended these observations by finding that the cellular exudate after an intrapleural or intraperitoneal injection in animals of fatty oil or oil emulsion of lecithin is almost entirely made up of the lymphoid type of cell.

It is well known that the local reaction following an injection of homologous living tissue in mice consists mainly of a lymphoid cell outpouring[4] similar to that described above. Murphy and Nakahara[5] observed that this local reaction is accompanied by evidences of increased proliferative activity among the lymphoblastic cells of the spleen and lymph nodes. It may be stated in passing that mice thus injected with homologous tissue become highly resistant to

[1] Ramond, M. F., *Compt. rend. Soc. biol.*, 1904, lvi, pt. 2, 95.

[2] Clark, E. R., and Clark, E. L., *Am. J. Anat.*, 1917, xxi, 421.

[3] Bergel, S., *Berl. klin. Woch.*, 1919, lvi, 915; *Z. exp. Path. u. Therap.*, 1920, xxi, 216; *Ergebn. inn. Med.*, 1921, xx, 36.

[4] Da Fano, C., *Z. Immunitätsforsch., Orig.*, 1910, v, 1.

[5] Murphy, Jas. B., and Nakahara, W., *J. Exp. Med.*, 1920, xxxi, 1.

transplanted cancer.[6] A like stimulation of the proliferative activity of the lymphoid cells may be induced by certain physical agents,[7] with resultant increased resistance to cancer transplants.[8]

In view of these observations it was regarded as of interest to determine whether or not the local reaction to oil is accompanied by a general lymphoid stimulation and, if so, the effect on the resistance to cancer inoculation in mice.

General Lymphoid Response to Injections of Olive Oil.

Commercial olive oil,[9] described as the first expression, was used in the following experiments. The injections were made intraperitoneally, followed by a histological study of the general condition of the lymphoid organs, with special attention to the number of mitotic figures present as this had been shown to be a fair index of the degree of stimulation.[10]

Experiment 1.—Twenty-five normal white mice of about the same age and size were divided into five groups of five mice each. The mice of Group A received an intraperitoneal injection of 0.1 cc. of the oil, Group B received 0.2 cc., Group C, 0.3 cc., Group D, 0.5 cc., and Group E, 0.7 cc. One animal from each group was killed for histological study 24 hours, 48 hours, 3 days, 4 days, and 5 days after the injection.

Group A.—Mice of this group received each 0.1 cc. of the oil. No unusual features were found in lymphoid organs of any of the mice, excepting one that was killed 4 days after the injection, and the germ centers of the lymphoid tissue of this mouse showed a marked increase of mitotic figures.

Group B.—Mice of this group received each 0.2 cc. of the oil. The mouse killed 24 hours after the injection showed no unusual condition, but four others killed at 48 hours to 5 days did show, particularly in germ centers (Fig. 1), a definite increase in mitosis.

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[6] Bashford, E. F., *Brit. Med. J.*, 1906, ii, 209.

[7] Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1919, xxix, 1. Nakahara, W., *J. Exp. Med.*, 1919, xxix, 17. Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1920, xxxi, 13.

[8] Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1919, xxix, 25. Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1921, xxxiii, 429.

[9] It was estimated that this product contained about 30 per cent of cottonseed oil. This was determined by the density of the brown color following the addition of nitric acid, using as a standard known dilutions of the cottonseed oil.

[10] Nakahara, W., and Murphy, Jas. B., *Anat. Rec.*, 1921, xxii, 107.

Group C.—Mice of this group received each 0.3 cc. of oil. An appreciable but less marked increase in the number of mitotic figures was present 48 hours to 5 days after the injection.

Group D.—Mice of this group each received 0.5 cc. of oil. Judged by the number of mitotic figures, proliferative activity was retarded in the 24 and 48 hour specimens and about normal in the remaining animals.

Group E.—Mice of this group each received 0.7 cc. of the oil. In mice killed at the 24 and 48 hour periods, the lymphoid organs, especially the spleens, were much reduced in size. These small lymphoid organs showed an almost complete suppression of mitosis, reduction in the amount of lymphoid tissue, and, in the splenic pulp, vacuoles of various sizes. At later periods, while the size of lymphoid organs was still small, the rate of mitosis was approximately normal.

The preceding experiment indicates that the most pronounced reaction in the lymphoid organs followed the intraperitoneal injection of a dose of 0.2 cc. of the oil.

Experiment 2.—Twelve normal white mice were injected intraperitoneally with 0.2 cc. of olive oil as in Group B of the preceding experiment. The mice were killed in groups of three each at 48 hour, and 4, 7, and 10 day intervals.

Of the three mice killed 48 hours after the injection, one showed unmistakable increase in the number of mitotic figures in the lymphoid germ centers, another a less striking but distinct increase, and a third mouse no appreciable increase.

At 4 and 7 day periods, all the mice showed a greatly increased number of mitotic figures. In one mouse killed at the 4 day period the mitosis was especially exaggerated, and was present not only in, but outside the germ centers.

At the 10 day period, mitotic figures were still abundant in two mice and there were a few in the third.

As a control to the above experiment, a number of normal mice were injected intraperitoneally with 0.2 cc. of liquid petrolatum without leading to an increase of the mitosis.

Other Organs.—The livers of a few of the mice injected with oil had many intracellular vacuoles suggestive of fatty inclusions. Also occasionally there were found a marked dilatation of the capillaries and sinus-like spaces of the suprarenal gland and an increase in the number of mitotic figures in the cortical cells (lymphocytes) of the thymus. These findings were of irregular occurrence and, therefore, should not be classed as typical changes induced by the oil injection. No special alterations were noted in the thyroid glands, kidneys, or bone marrow.

Cytology of the Peritoneal Exudate.—Smears were taken of peritoneal fluid at autopsy in Experiments 1 and 2. As already pointed out by Bergel,[3] all the smears showed numerous cells of lymphoid group, including typical large and small lymphocytes, so called transitional cells, plasma cells, large cells resembling macrophages, and true endothelial cells. Morphologically, there is a complete series of intergradations from the typical small lymphocyte to the large macrophage-like cell, suggesting that they all belong to a single biologic group, a point which has been emphasized by Bergel.[3] At an early stage (24 hours after the oil injection), there is a considerable number of polymorphonuclear cells, particularly neutrophils and eosinophils, in addition to lymphoid cells, but the number of these granular cells soon falls off. At the 48 hour period, the lymphoid reaction is apparently at its height, while at this time the polymorphonuclear reaction has about subsided (Fig. 2). The local reaction occurs regardless of the amount of oil injected and lasts as long as the oil remains in the peritoneal cavity. When 0.2 cc. of olive oil was injected the reaction tended to subside within 10 days, or hand in hand with the gradual absorption of the oil.

The above experiments indicate that lymphoid tissue as a whole responds definitely to an intraperitoneal injection of olive oil, which, if given in the optimum quantity, brings about a marked stimulation of the proliferative activity of this tissue. Studies on the peritoneal exudates, moreover, confirmed the results reported by Ramond,[1] Clark,[2] and Bergel,[3] regarding the local cellular manifestations about the injected fatty and lipoidal substances.

The relation between the local lymphoid reaction and the stimulation of the germ centers cannot be determined directly. However, in view of the lipolytic function of lymphoid cells,[11] it does not seem improbable that the local lymphoid response to the injected oil is an expression of the attempt of the body to dispose of the injected material. If so, it is conceivable that an optimum grade of activity on the part of lymphoid cells may lead to the general lymphoid stimulation.

[11] Bergel, S., *Münch. med. Woch.*, 1909, lvi, 64. Fiessinger, N., and Marie, P.-L., *Compt. rend. Soc. biol.*, 1909, lxvii, pt. 2, 177.

Cancer Inoculation Experiments.

The production by injection of olive oil of lymphoid stimulation, essentially similar to the condition of induced potential immunity to transplanted cancers, suggested the possibility of rendering animals resistant by the same method. Experiments were accordingly undertaken to determine this point.

The dose of olive oil was given in a single intraperitoneal injection. Cancer inoculation was made subcutaneously in the left groin, and the rate of growth of tumors was charted thereafter at weekly intervals for 3 weeks. The strain of cancer used was Bashford Adenocarcinoma No. 63, and all the mice were young adults of white variety from the same stock.

The Degree of Resistance in Relation to the Amount of Olive Oil Injected.

Experiment 3.—In order to determine the optimum dose of olive oil, this substance was injected into mice in different quantities, ranging from 0.1 to 0.7 cc. Cancer inoculation was made in every case 10 days after the injection. The results are summarized in Table I.

TABLE I.

Experiment 3.

Amount of olive oil.	Treated mice.		Controls.	
	Resistance.	No. of mice.	Resistance.	No. of mice.
cc.	*per cent*		*per cent*	
0.1	20.5	19	0.0	10
0.2	40.0	18	0.0	19
0.3	25.0	20	5.5	21
0.5	6.1	23	11.1	9
0.7	0.0	9	0.0	9

Since the number of mice in each group is small, slight differences in the percentage of resistance in the several groups should not be considered as significant. However, the fact that emerges is that by injecting 0.2 cc. of olive oil 10 days before giving a cancer inoculation, mice are rendered more resistant to the inoculated cancer than they

normally are. This same point was brought out in three additional experiments, the results of which are shown in Table II.[12]

TABLE II.

Experiments 4 to 6.

Experiment No.	Mice injected with olive oil 10 days before cancer inoculation.		Controls.	
	Resistance.	No. of mice.	Resistance.	No. of mice.
	per cent		*per cent*	
4	43.7	16	11.1	9
5	52.6	19	10.0	10
6 (Text-fig. 1).	41.0	20	10.5	19

The Degree of Resistance in Relation to the Time of Cancer Inoculation.

Experiment 7.—In the preceding experiment the cancer inoculations were made 10 days after the oil injection. In order to ascertain the period at which the maximum degree of resistance is manifested, the inoculations in the following experiment were made at various intervals after an injection of 0.2 cc. of the oil. The results are shown in Table III.

TABLE III.

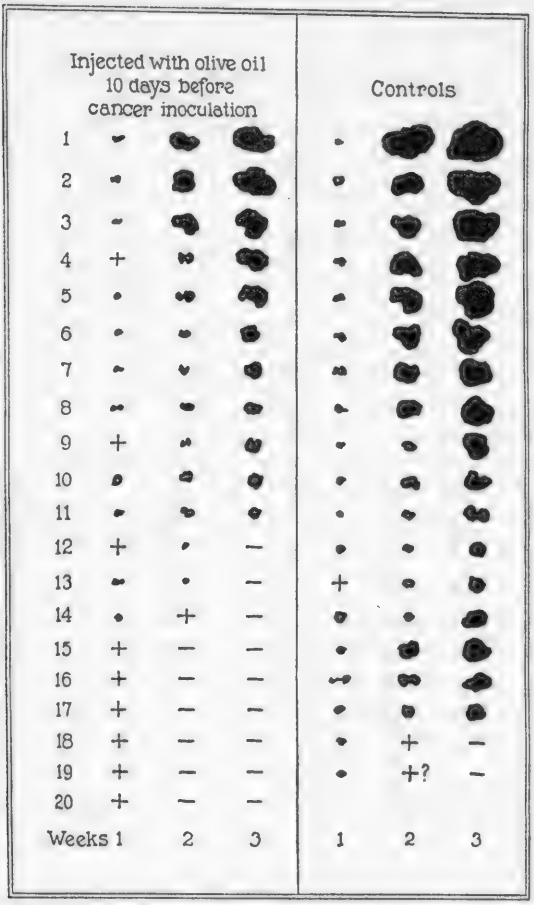
Experiment 7.

Intervals between oil injection and cancer inoculation.	Resistance.	No. of mice.
days	*per cent*	
5	20.0	10
10	44.4	9
15	30.0	10
25	10.0	10
Control.	0.0	12

In all the former types of induced resistance to transplanted cancer so far studied, there is a period following the treatment during which there is slight, if any, evidence of resistance. This is not only true as

[12] The amount of olive oil should be slightly changed according to the size of the mouse. For a large mouse, weighing over 25 gm., as much as 0.3 cc. can be given.

regards the injection of homologous living tissue,[13] but also equally after exposure to intense dry heat and after small doses of x-rays.[14]



TEXT-FIG. 1. Experiment 6. The rate of growth of Bashford Adenocarcinoma No. 63 in mice injected with 0.2 cc. of olive oil 10 days before inoculation, contrasted with the rate of growth in untreated mice.

[13] Bashford, E. F., Murray, J. A., and Cramer, W., *Proc. Roy. Soc. London, Series B*, 1907, lxxix, 180. Woglom, W. H., *J. Exp. Med.*, 1912, xvi, 629.

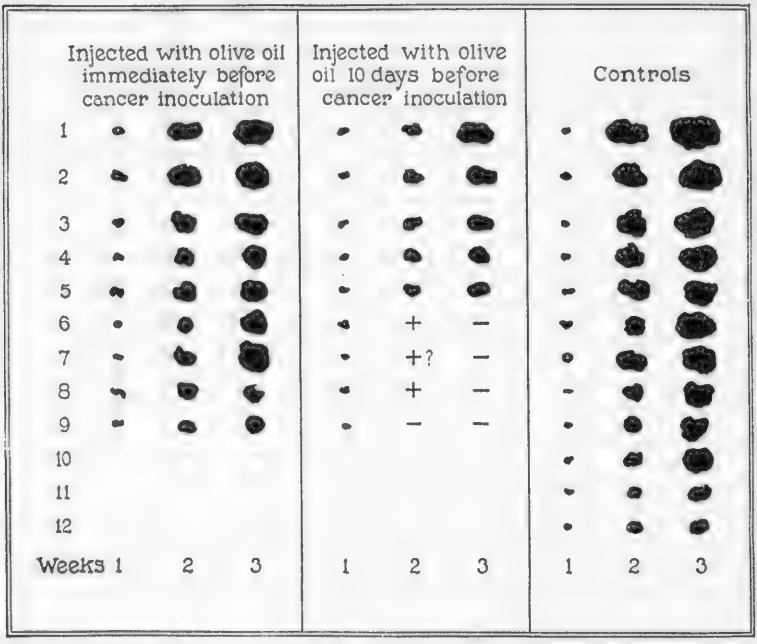
[14] Murphy, Jas. B., *Proc. Nat. Acad. Sc.*, 1920, vi, 35. Murphy, Jas. B., Nakahara, W., and Sturm, E., *J. Exp. Med.*, 1921, xxxiii, 423.

TABLE IV.

Experiments 8 to 10.

Experiment No.	Resistance.		
	Group A.*	Group B.	Group C.
8 (Text-fig. 2).	44.4 per cent (9 mice).	0.0 per cent (9 mice).	0.0 per cent (12 mice).
9	30.0 " " (10 ").	10.0 " " (10 ").	0.0 " " (10 ").
10	50.0 " " (8 ").	10.0 " " (10 ").	12.5 " " (8 ").

* Group A was made up of mice injected with the oil 10 days before the cancer inoculation. Group B mice were injected with the oil immediately before the inoculation. Group C comprised the control mice receiving no oil and inoculated with the same tumor.



TEXT-FIG. 2. Experiment 8. Effect of 0.2 cc. of olive oil on the rate of growth of Bashford Adenocarcinoma No. 63, when administered 10 days and immediately before inoculation.

That a similar state arises after an injection of olive oil is shown by the above experiment. A more complete test of this point is given in Table IV.

Other Oils.—Several other oils of different chemical constitution have been tested; namely, cod liver oil, cocoanut oil, sperm oil, and liquid petrolatum (Nujol). Cancer inoculations made 10 days after injection of 0.2 cc. of these several oils induced no appreciable resistance. The tests do not, however, exclude the possibility of a suitable dosage of these oils inducing a result comparable to that given by olive oil.

Histological Changes Accompanying the Resistance Induced by Olive Oil.

The following experiments were carried out in order to supply material for a histological study of the nature of the reaction accompanying the resistant state induced by olive oil.

Experiment 11.—Ten normal white mice were given an intraperitoneal injection of 0.2 cc. of olive oil each. 10 days later they were inoculated with fragments of a Bashford Adenocarcinoma No. 63, subcutaneously in the left groin. The mice were then killed in pairs 24 hours, 48 hours, 3 days, 4 days, and 5 days after the inoculation and the grafts and the lymphoid organs were removed for histological study.

Local Cellular Infiltration.—The occurrence of a characteristic exudate around the cancer grafts in resistant animals has long been known.[15] This local reaction, in which the cells of lymphoid variety take a prominent part, subsides rapidly after the necrosis of the grafts has become complete. On this account the grafts in the present experiment were removed at early periods.

Specimens taken 24 and 48 hours after inoculation showed various types of wandering cells, especially polymorphonuclear leucocytes and fibroblasts, collecting in a great number around the graft. At the 3 day period, however, much of the polymorphonuclear reaction had subsided and there was a marked infiltration of lymphocytes, plasma cells, and fibroblasts (Figs. 3 and 4) closely resembling the local re-

[15] Burgess, A. M., *J. Med. Research*, 1909, xxi, 575. Rous, P., and Murphy, Jas. B., *J. Exp. Med.*, 1912, xv, 270. Tyzzer, E. E., *J. Med. Research*, 1915, xxxii, 201.

action known to occur in cancer-resistant animals. Cell infiltration similar to the latter but in varying amounts was encountered in all the specimens taken at 4 and 5 day periods.

Stimulation of Lymphoid Tissue.—Animals resistant to cancer inoculation tend to develop on inoculation lymphoid hyperplasia.[16,17] Murphy and Nakahara[5,17] have shown that this phenomenon, which is characterized by a marked increase in the number of mitotic figures in germ centers of lymphoid tissue, occurs very soon after cancer grafting in potentially resistant animals.

Spleens and lymph nodes taken as early as 24 hours after cancer inoculation in the above experiment showed that the number of mitotic figures in lymphoid tissue was greater than is seen in normal animals. At the 48 hour and 3 day periods the reaction appears to reach its height and at the latter periods mitotic figures were found in great-numbers in the germ centers of the spleen (Fig. 5) and lymph nodes, and often in considerable numbers even in the lymph cord of the node (Fig. 6). It should be stated that one animal each of the 4 and 5 day period failed to show any increase of mitotic figures, an irregularity without significance.

Blood Lymphocytosis.—Murphy and his associates have shown that a marked increase in the number of circulating lymphocytes accompanies the state of resistance to transplanted cancer.[18] In order to ascertain whether or not such a lymphoid crisis occurs after cancer inoculation in the mice treated with olive oil, white cell counts were made of a number of such mice.

Experiment 12 (*Text-Fig. 3*).—Nineteen normal white mice were injected intraperitoneally with 0.2 cc. of olive oil and cancer inoculation was made in all of the mice 10 days afterward. Ten of the mice proved to be resistant and nine susceptible to the inoculation.

The average number of lymphocytes per c.mm. of blood in the resistant mice, 1 day before the oil injection, was about 4,800 and of polymorphonuclear leucocytes about 4,700. 3 days after cancer inoculation the lymphocytes increased

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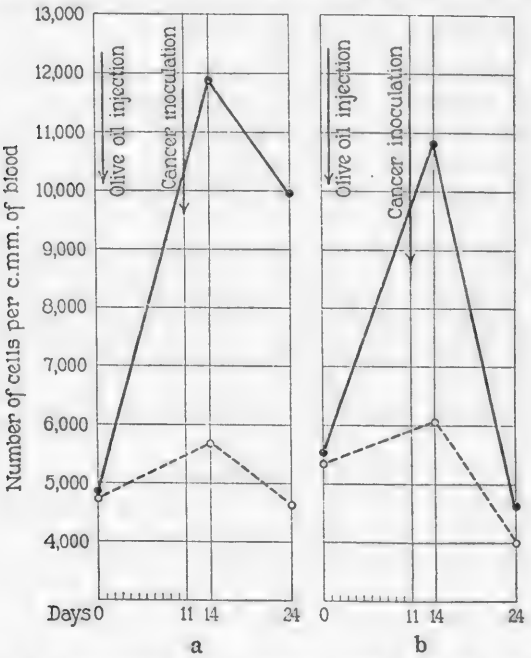
[16] Mottram, J. C., and Russ, S., *Proc. Roy. Soc. London, Series B*, 1917–18, xc, 1.

[17] Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1921, xxxiii, 327.

[18] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 204. Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1921, xxxiii, 433.

to about 11,800, while the polymorphonuclear cells were only slightly increased, being about 5,600. 2 weeks after cancer inoculation the lymphocyte count was still high, being about 10,000, while the polymorphonuclear leucocytes had returned to the initial level (about 4,600).

In cancer-susceptible mice, 1 day before the oil injection, the average number of lymphocytes and polymorphonuclear leucocytes was about 5,500 and 5,400



TEXT-FIG. 3. Experiment 12. Composite curves of white cell counts on mice injected with 0.2 cc. of olive oil and inoculated with Bashford Adenocarcinoma No. 63, 10 days later. (*a*) Composite curves from ten mice proved to be immune; (*b*) composite curves from nine mice proved to be susceptible. ——— Lymphocytes. ..... Polymorphonuclears.

respectively. The counts were quite high 3 days after the inoculation, the lymphocytes being about 10,800 and the polymorphonuclear leucocytes about 6,000. At the end of the 2nd week after the cancer inoculation, all the cells were much reduced, the lymphocytes being about 4,600 and polymorphonuclear leucocytes about 4,000.

Experiment 13.—An experiment similar to the preceding one was carried out with ten mice, five of which were resistant and five susceptible.

In the resistant mice the average number of lymphocytes per c.mm. of blood 1 day before the oil injection was about 4,000; polymorphonuclear leucocytes about 4,400. 2 weeks after the cancer inoculation the lymphocytes were a little over 8,000 while the polymorphonuclear leucocytes had decreased to about 3,400.

In the susceptible mice, 1 day before the oil injection the lymphocytes were about 3,600 and the polymorphonuclear leucocytes about 4,000. 2 weeks after cancer inoculation the lymphocytes were unchanged and the polymorphonuclear leucocytes had perceptibly increased (about 5,700).

The preceding experiments show that a characteristic lymphoid crisis occurs in the blood during the establishment of resistance to cancer grafting induced through olive oil injection. Experiment 12 suggests that even in mice that proved to be susceptible there is apparently an inadequate reaction, which, however, is not of long duration.

It should also be stated that the injection of olive oil alone does not bring about a lymphocytosis. White cell counts were made on ten normal mice injected with 0.2 cc. of olive oil. In certain of the mice there was an increase and in others a decrease in number of circulating lymphocytes during the first 10 days after the oil injection, but the changes were too slight and too irregular to be of importance.

DISCUSSION.

The early work of Bashford and his coworkers[6] established the fact that resistance to transplanted cancer in mice could be induced by the inoculation of homologous living tissues. Later Murphy and his associates[14] showed that resistance could be induced by the use of suitable doses of x-rays and intense dry heat. The experiments reported in this paper demonstrate that resistance may be induced by still another means; namely, by the intraperitoneal injection of olive oil. Thus it may be said that resistance to transplanted cancer can be induced by three classes of agents—homologous tissue, a biological agent; x-rays and heat, physical agents; and olive oil, a chemical agent.

There is little direct indication concerning the nature of the common factors responsible for the resistant state induced by these various agents but the manifestations associated with phenomena of resistance are the same regardless of the means used to induce this state. These associated manifestations are, a latent period after the treatment, during which time there is no evidence of resistance,

a local cellular reaction about the inoculated cancer graft, an increase in the number of circulating lymphocytes, and a marked increase in the proliferative activity in the lymphoid organs. The indirect evidence associating the lymphoid cell with the mechanism of resistance to cancer is so strong as to leave little doubt that this cell has an important, if not the most important rôle in bringing about the resistant state.

SUMMARY.

The experiments reported in this paper show that it is possible to render mice resistant to transplanted cancer by injections of a suitable quantity of olive oil. In the course of the development of the resistance a definite period of latency is detectable following the oil injection, and the maximum degree of resistance appears at about the 10th day. This state of resistance, as has been determined by histological studies, is preceded by a proliferation of the cells of the lymphoid germ centers and, after the cancer inoculation, is associated with a lymphoid infiltration about the grafts, as well as by a second stimulation of the lymphoid germ centers and an increase in the number of the circulating lymphocytes.

EXPLANATION OF PLATES.

PLATE 34.

FIG. 1. Germ center of spleen 4 days after an intraperitoneal injection of 0.2 cc. of olive oil. *M*, mitotic figure.

FIG. 2. Peritoneal exudate 48 hours after an intraperitoneal injection of 0.2 cc. of olive oil.

PLATE 35.

FIG. 3. 48 hour old cancer grafts and surrounding connective tissue in mouse injected with 0.2 cc. of olive oil 10 days previous to cancer inoculation. Note the extensive cellular infiltration around the graft.

FIG. 4. High power view of an area of infiltration in the above specimen, showing the types of cells participating in the infiltration.

PLATE 36.

FIG. 5. Germ center of spleen of mouse injected with 0.2 cc. of olive oil and inoculated with cancer 10 days later. 3 days after cancer inoculation. *M*, mitotic figure.

FIG. 6. Medulla of lymph node of mouse treated similarly to that of Fig. 5. 3 days after cancer inoculation. *M*, mitotic figure.

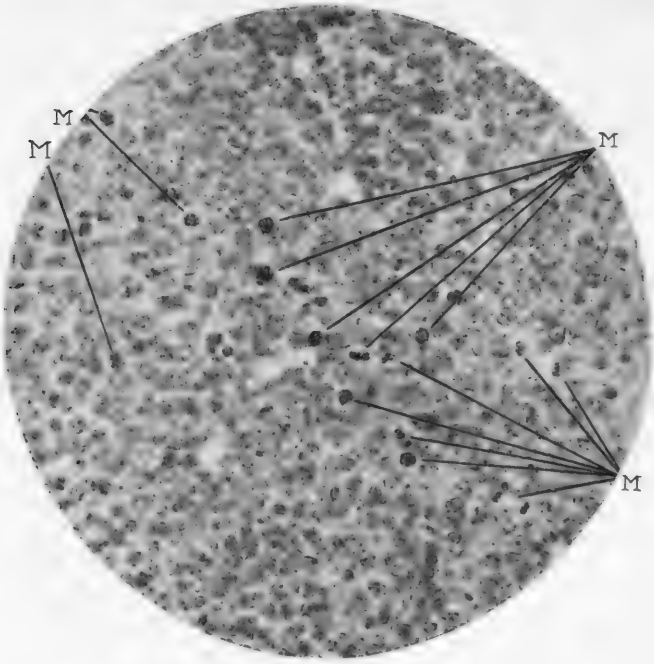


FIG. 1.

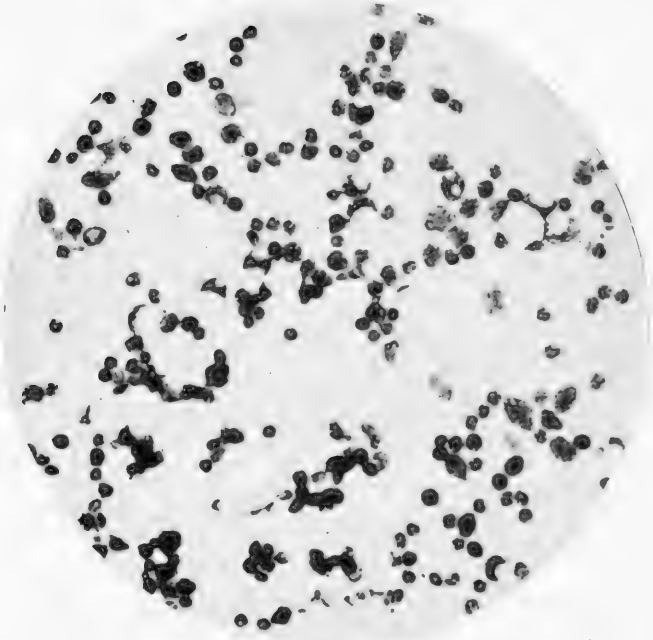


FIG. 2.

(Nakahara: Lymphoid activity. VI.)

FIG. 3.

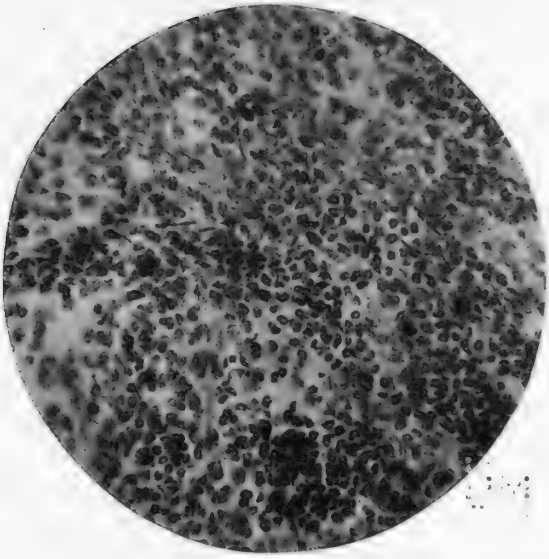


FIG. 4.

(Nakahara: Lymphoid activity. VI.)

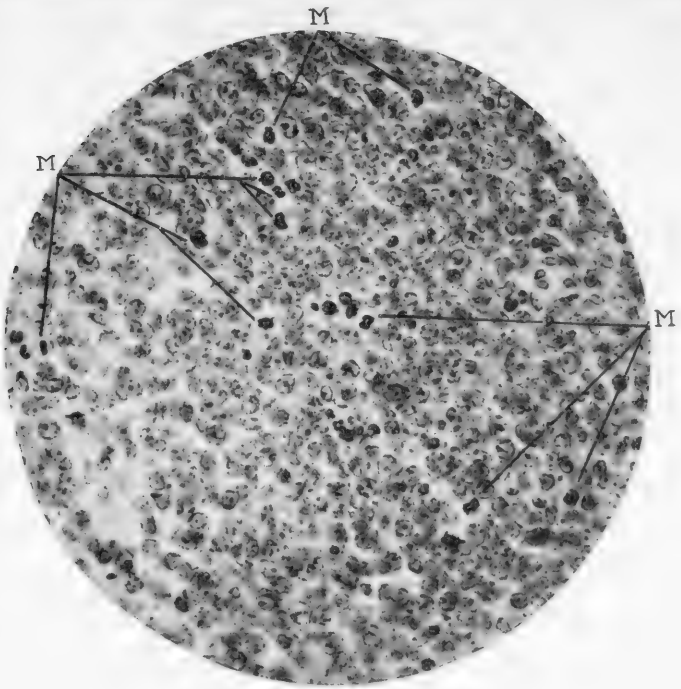


FIG. 5.

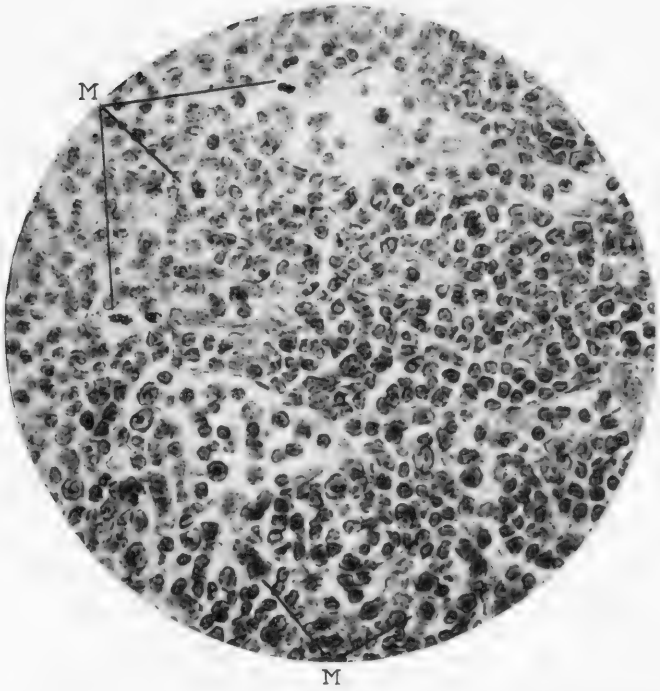


FIG. 6.

(Nakahara: Lymphoid activity. VI.)

THE ERYTHROPOIETIC ACTION OF GERMANIUM DIOXIDE.

II. The Source of the Erythrocythemia Produced by Germanium Dioxide in the Albino Rat.

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(*From The Wistar Institute of Anatomy and Biology, Philadelphia.*)

(Received for publication, November 9, 1921.)

In a previous publication it has been shown that germanium dioxide is non-toxic to the mature female albino rat when injected subcutaneously in amounts up to 180 mg. per kilo of body weight. This is a quantity equivalent to a dose of nearly 11 gm. for a 60 kilo man. The measure of the toxicity was survival after the administration of the compound. In no case did any animal receiving the germanium dioxide succumb or even present obvious symptoms of disturbance.[1]

A systematic study was then made of the effect of the compound on the erythrocyte and white cell content of the normal rat blood.[2] It was found that, regardless of sex, a marked and valid increase in the red cells in the circulation followed the injection of relatively small amounts of germanium dioxide solutions. In view of certain gross findings at autopsy of the rats used in these experiments, and because of the persistence of the effect for many days after the injections, we considered ourselves justified in concluding that germanium dioxide is an erythropoietic agent of remarkable potency.

In this report we propose to present the completed evidence on which that belief is based. The material used was obtained from the rats serving as subjects in the preceding paper.[2]

[1] Hammett, F. S., Müller, J. H., and Nowrey, J. E., Jr., *J. Pharmacol. and Exp. Therap.*, 1922 (in press).

[2] Hammett, F. S., Nowrey, J. E., Jr., and Müller, J. H., *J. Exp. Med.*, 1922, xxxv, 173.

A review up to 1915 of the data and literature concerned with the phenomenon of erythrocythemia has been published by Lamson.[3]

The cause of any given erythrocythemia may be apparent, such as a decrease in the plasma volume, or real, as, for example, a production of new cells, the division of red cells, or a release from deposits. Lamson in studying the erythrocythemia produced by the injection of epinephrine came to the tentative conclusion that the imbibition of fluid by the liver and consequent decrease in plasma volume is a factor of considerable importance.[4] However, since Lamson and Keith[5] have reported that the plasma volume and the erythrocyte content of the blood may vary independently of each other, it would appear that their theory has yet to be proved by further experimentation. Because of the incompleteness of the evidence, and in view of our studies of the bone marrow of rats treated with germanium dioxide, we consider that, for the present at least, the question of the relation of the plasma volume to the germanium erythrocythemia is of relatively minor importance.

Similar considerations apply to the question of the possibility of the observed erythrocythemia arising from either a division of red cells already in the circulation or by release from deposits within the organism.

It may be noted here that the germanium dioxide erythrocythemia differs from that produced by Lamson,[6] with epinephrine in that it is lasting in nature, while that following epinephrine is but transitory. While it is true that we have records of two rats with a persistent red cell increase 5 weeks after a single injection of germanium, and of eleven others showing an erythrocythemia 11 and 14 days after the last injections, yet the objection can be raised, and justly, that this persistence of the effect may well be due to a gradual release of the stimulating compound from a protoplasmically bound condition arising at the time of injection. This would imply a marked stimulating power by relatively minute amounts of germanium. Certain data,

[3] Lamson, P. D., *J. Pharmacol. and Exp. Therap.*, 1915, vii, 169.

[4] Lamson, P. D., *J. Pharmacol. and Exp. Therap.*, 1920–21, xvi, 125.

[5] Lamson, P. D., and Keith, N. M., *J. Pharmacol. and Exp. Therap.*, 1916, viii, 247.

[6] Lamson, P. D., *J. Pharmacol. and Exp. Therap.*, 1916–17, ix, 129.

however, indicate that, in man at least, it is necessary to administer a certain minimum amount before a detectable rise in red cells is obtained. This amount is in excess of what might be considered to be released from the tissues during the persistence of effect observed in our studies, per unit of time. This point, however, can only be decided by investigation.

If the erythrocythemia produced by germanium dioxide is actually the result of an erythropoietic action of the compound, evidence should be found in one or all of the blood-forming organs—spleen, liver, and bone marrow—of a stimulation to new red cell formation. Also young erythrocytes should be found in the circulating blood.

Gross inspection of the tissues at autopsy showed that the livers of the germanium-treated rats were of a reddish purple color, while the livers of the controls were a tobacco-brown. The spleen in the rat is normally a very dark red, and no differences in the appearance of this organ in the two groups could be detected. When the marrow of the long bones was exposed it was seen that in the controls this tissue was light coffee color while that of the test animals was maroon.

Small segments of the liver and the spleen were cut from as near the same locality as possible from these organs in all the rats, tests and controls, and were fixed in formaldehyde. The femurs were removed, cracked open so as to expose the marrow, fixed in formaldehyde, and carried through the dehydrating process to absolute alcohol. The marrow was then removed from as near the middle portion of the shaft of the bone as possible and was transferred to chloroform with the other tissues and embedded in paraffin. The animals of any given series were all autopsied within a few minutes of each other, and the fixation, dehydration, and embedding were carried on simultaneously so that errors due to differences in technique would be eliminated.

The sections of the livers and spleens were stained with hematoxylin and eosin. Methylene blue and eosin, and Goodpasture's polychrome stain, without the preliminary permanganate and oxalic acid treatment, were used for the bone marrow.

The sections of the livers of the germanium-treated rats showed no evidence of the resumption by this organ of its original erythropoietic function. There was present, however, in most cases an apparent dilatation of the hepatic capillaries, in which were many erythrocytes

but no nucleated red cells. This relative engorgement of the liver with red corpuscles which did not occur in the controls—and which explains the gross findings at autopsy—rather effectively speaks against the idea that the erythrocythemia is due to liberation from the liver of red cells that might be considered to be held therein, particularly since in the control livers the absence of erythrocytes in the capillaries is noticeable. In fact the increased number of red cells in the livers of the test rats might give rise to the opinion that the actual number of circulating cells observed was not a complete index of the new red cell formation. However that may be, it is certain that, in so far as these observations go, the liver is not the organ causing the erythrocythemia, although it is in some way as yet unknown affected directly or indirectly by the administration of the germanium dioxide.

Study of the sections of the spleens from the test animals also shows no evidence of the taking on of the original function of red cell formation. Nor are there any indications of an increased red cell destruction by the splenic phagocytes. Whether or not an increased red cell destruction accompanies the germanium erythrocythemia can only be determined by investigation. The impression is given that there is a slightly greater erythrocyte congestion in the sections of this tissue from the germanium-treated rats as compared with the controls, but it is not marked enough to be decisive. There also seems to be a more dense concentration of cells in the Malpighian corpuscles of the spleens of the test rats. However, this too is not sufficiently definite to be decisive. It is thus evident that the spleen is not the source of the increased red cell content of the circulating blood, although possibly the content is affected in some way or other, either directly or indirectly, by the administration of germanium dioxide.

In the sections of bone marrow of the germanium-treated rats there was ample evidence that the compound had produced a stimulation in formation of new red cells in that tissue over and above that present in the marrow of the controls. Not only were there more centers where the nucleated erythrocytes were in evidence, but there were more of these types of cells per unit area. Counts were made of the nucleated red cells in twenty fields, under the oil immersion lens, of longitudinal sections cut through the greatest diameter of the pieces

TABLE I.

Counts of the Nucleated Erythrocytes of the Bone Marrow.

Series No.	Sex.	Controls.	Test rats.			
			No. 1.	No. 2.	No. 3.	Mean.
2	Males.	187	242	232		237
	Females.	231	532	620	524	559
3	Males.	254	476	545	611	544
	Females.	308	477	448	492	472

removed from the bones. The field for counting was defined by an eyepiece micrometer. The results are given in Table I. They demonstrate conclusively that the erythrocythemia produced by the injection of germanium dioxide solution takes its origin from a stimulation of erythrocyte formation in the bone marrow.

With such a marked erythrocyte formation one would expect to find evidence in the circulating blood of young red cells. In fact, the presence of such cells in the blood would be a satisfactory confirmation of the bone marrow findings. In none of the rats used in these experiments, tests or controls, could there be found in the blood any cells that could be identified as nucleated erythrocytes. Nevertheless, in the smears from both the controls and the germanium-treated rats there were many polychromatic staining erythrocytes,

TABLE II.

Relative Number of Polychromatic Staining Erythrocytes in the Smears.

	Preliminary period.		During germanium administration.	
	Controls.	Test rats.	Controls.	Test rats.
Mean	49.0	40.0	59.0*	79.0
Standard deviation	6.4	16.2	12.9	17.6
Probable error of mean	1.6	2.4	2.8	2.1

* This slight increase which is by no means comparable with that obtained with the test animals confirms the slight rise in erythrocytes in these animals reported in the preceding paper.[2]

or young red cells. In order to determine whether or not there were more of these cells in the blood of the test animals as compared with the controls, a systematic count was made of their occurrence in the smears made for the differential leucocyte counts. The results are given in Table II. They represent the means, the standard deviations, and the probable error of the means of all the smears. It should be noted that every time the blood was taken for counting as reported in the preceding paper[2] smears were also taken for this purpose.

This table shows that there is a statistically valid increase in the number of erythrocytes taking the polychromatic stain in the blood of the germanium-treated rats. This confirms the bone marrow findings.

For purposes of completeness differential counts were made on the smears of all the rats. The normal values are to be published elsewhere. A comparison of the distribution of the white cells of the test rats during the preliminary period with that occurring during the germanium administration and with the controls showed that no changes occurred of sufficient magnitude to be significant.

SUMMARY AND CONCLUSIONS.

A histological comparison of the liver, spleen, bone marrow, circulating young erythrocytes, and differential count in mature male and female albino rats receiving germanium dioxide with their litter controls not receiving this compound was made.

It was found that the livers of the test animals in most cases showed a condition of capillary dilatation and that more erythrocytes were in these capillaries than were in those of the controls. There was no evidence of any red cell formation by the liver.

The spleens of the test rats gave the impression of being slightly more congested and of having a slightly more dense concentration of cells in the Malpighian corpuscles than those of the controls. There was no evidence of an increased red cell destruction nor was there any evidence of splenic erythropoiesis.

In the bone marrow of the rats which had received the germanium dioxide injections there was evidence of a marked stimulation in formation of nucleated erythrocytes, in that many more of these cells were found here than in the marrow sections of the controls.

The circulating blood of the test rats contained more young red cells as demonstrated by the increased number of erythrocytes taking the polychromatic stain than did the blood of the controls.

No noteworthy differences in the values for the various types of white cells in the circulation determined by the differential count could be found between the two groups.

Using, then, as an acceptable criterion of erythropoiesis an increase in the number of erythrocytes in the circulation which is accompanied by an increase in the number of young red cells, and an increased number of nucleated erythrocytes in the bone marrow, we consider ourselves justified in concluding that germanium dioxide is a potent erythropoietic agent and the source of the erythrocythemia produced by this compound is the increased production of red cell precursors by the bone marrow stimulated to increased activity by the compound used.

THE EFFECT OF FLOOD DIURESIS ON HEMOGLOBINURIA.

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(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, November 9, 1921.)

The fact is well recognized that a considerable quantity of hemoglobin must be free in the plasma if any is to pass the renal barrier and appear in the urine. The pigment is, like dextrose, a "threshold substance." It readily penetrates into the renal tubules but is absorbed again more or less completely during its course through them.[1] This being true, diuresis should diminish the chances of absorption by hastening the flow of fluid, and tend to lead to the appearance of the pigment in the urine. Evidence will here be presented that such is the case. Hemoglobinuria, like glycosuria, is much favored by flood diuresis.

Method.

A concentrated solution of hemoglobin was abruptly thrown into the circulation of rabbits and dogs, followed in some instances by a slower injection of salt solution. The amount of pigment introduced was slightly less than that required to produce hemoglobinuria in the absence of diuresis. The urine was collected at intervals by catheter.

All of the animals were males. Individuals were selected with normal kidneys, as indicated by the general character of the urine and proven by the autopsy findings.

Great care was necessary to prevent hemorrhage during the catheterization of the rabbits, and despite it a few red cells were frequently encountered afterwards in the urine. For this reason the experiments were repeated on dogs, in which the complication can be avoided. The animals were stretched out, the bladders emptied by catheter as completely as possible, and the urine set aside for

[1] Adami, J. G., *J. Physiol.*, 1885, vi, 382.

TABLE 1.

Influence of Flood Diuresis on Hemoglobinuria.

Experiment No.	Rabbit.	Weight.	Hemoglobin of blood.	Per cent of total hemoglobin injected.	Amount injected.	Followed by salt solution.	Urine of next ½ hr.				Remarks.
							Amount.	Color.	Guaiac test.	Spectroscopic bands.	
		gm.	*per cent*		*cc.*	*cc.*	*cc.*				
Rabbits.											In Experiment 1, the bloods of the animals were assumed to have the same percentage of hemoglobin, and in consequence the amounts of pigment injected differed directly as the body weight.
1	Control.	3,050	61.5	0.8	1.68	—	3.0	Yellow.	0	Absent.	
	Diuresis.	2,575	66.5	0.8	1.42	40	26.0	Pink.	+++	Present.	
2	Control.	3,050	71.0	0.8	1.9	—	4.0	Straw.	Tr.	"	
	Diuresis.	2,725	75.0	0.8	1.8	20	8.0	Pink.	+++	"	
3	Control.	2,350	65.0	0.8	1.3	—	1.5	Straw.	0	Absent.	
	Diuresis.	2,450	73.0	0.8	1.6	20	8.0	Pink.	+++	"	
Dogs.	Time.										
1	—	7,250	72.0	1.6	5.8	—	2.5	Yellow.	0	"	
	Next day.			1.6	5.8	80	18.0	Orange-pink.	+++	Present.	
2	—	5,750	105.0	1.6	15.8	100	93.0	Pinky yellow.	+++	"	A rather dilute solution of hemoglobin was employed.
	2 days later.			1.6	15.8	—	15.5	Yellow.	0	Absent.	

3	—	7,250	73.0	1.8	9.6	40	22.0	Port wine.	+++	Present.	A different specimen of hemoglobin was used in this first observation from that employed in the three that followed.
	7 days later.			1.8	5.75	—	12.0	Yellow.	0	Absent.	
	After 5 days more.		82.0	1.8	6.5	—	9.0	"	0	"	$\frac{1}{60}$ gr. of morphine sulfate given prior to these observations.
	After 3 days more.			1.8	6.5	100	64.0	Orange-pink.	+++	Present.	

The urines were all negative for hemoglobin prior to the injections; and at no time were red cells found.

test, and the injection made into an ear vein. A hemoglobin solution prepared by the method of Sellards and Minot[2] from the blood of rabbits was rapidly injected and followed in many instances by warmed 0.9 per cent salt solution. To rule out all possibility of a mistake in the composition of the latter solution such as might, by intravascular laking, cause hemoglobinuria, its effect was tested regularly on an erythrocyte suspension.

A table will be used to present the results (Table I). The amount of hemoglobin injected is there recorded in percentages of the total quantity of the pigment already in circulation, as calculated from the percentage in the blood (Palmer method) and the total blood quantity, assuming this to constitute 5.5 per cent of the body weight in the rabbit and 8 per cent in the dog. Trial showed that in the rabbit one can inject hemoglobin up to at least 0.8 per cent of the body quantity without the appearance of any in the urine, save when diuresis is induced. In the dog 1.6 to 1.8 per cent gives similar results. No attempt was made to determine the normal renal thresholds more closely. In each experiment with rabbits two individuals received the same preparation of hemoglobin in identical proportion, one animal serving as control while in the other diuresis was induced by the injection of 20 to 40 cc. of saline solution given over a period of several minutes. In the work with dogs, each animal served as its own control, receiving repeated injections.

Catheterization was done just prior to each injection and ½ hour after it. The guaiac test and the spectroscope were used to disclose hemoglobin, but in many instances more striking testimony was obtained in the bright pink or red hue of the urine. To determine whether hemorrhage had been a factor, erythrocytes and their shadows were sought for in the urinary sediment.

Nine pairs of rabbits were used, each pair closely matched as regards size and blood condition. In two pairs which received 0.6 per cent and 0.8 per cent of hemoglobin, the urine of all of the animals remained free from the pigment; but only a questionable diuresis had been induced. In a third pair diuresis yielded a red urine, whereas that from the control animal was pink; but both specimens showed fairly numerous erythrocytes. In the remaining six experiments diuresis regularly resulted in a pink or red urine containing much hemoglobin and free from red cells or shadows, in contrast to the specimens from the controls, which, though sometimes showing a few red cells, were always yellow and only once yielded a positive guaiac reaction after the cells had been removed. Three such sharp-cut instances are presented in the table.

Four dogs were used. In one the results were relative only, hemoglobin appearing in quantity when diuresis was induced, and to a less extent next day when diuresis was lacking after the pigment injection. The findings in the remaining three animals have been tabulated (Table I). It will be seen that diuresis regularly resulted in hemoglobinuria, whereas without it none occurred.

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[2] Sellards, A. W., and Minot, G. R., *J. Med. Research*, 1917–18, xxxvii, 161.

By varying the order of the experiment, an overloading of the organism with pigment was ruled out as responsible for the results.

It is evident that flood diuresis markedly influences the elimination of hemoglobin into the urine, lowering the renal threshold for the substance as it does that for dextrose. According to Cushny[3] flood diuresis probably never occurs in man as a result of fluid taken by mouth. The tubules of the rabbit kidney are much less active in resorption than those of human beings; yet when very large quantities of water are administered to this animal "even its feeble power of absorption is sufficient to save the optimal fluid."[3] The present findings, then, have no bearing on the occurrence of clinical hemoglobinuria. They are not without significance, however, for a proper understanding of the renal siderosis that occurs in diseases which involve the repeated liberation of small quantities of blood pigment into the circulation.

CONCLUSIONS.

Flood diuresis so far lowers the renal threshold for hemoglobin that the pigment appears in quantity in the urine as result of a hemoglobinemia insufficient under ordinary circumstances to lead to the elimination of even a trace of it. In pathological conditions that involve blood destruction hemoglobin probably passes into the tubules much more often than it reaches the urine, being prevented therefrom by the resorptive activity of the tubular epithelium.

[3] Cushny, A. R., The secretion of the urine, London, New York, Bombay, Calcutta, and Madras, 1917, 145.

SIGNIFICANCE OF THE HEMOSIDEROSIS OF PERNICIOUS ANEMIA.

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The view that pernicious anemia is due to an injurious agent derived from the gastrointestinal tract has of late years attained the proportions of a doctrine, and it has determined the direction of all recent efforts to find the cause of the disease. Whether such a view is inevitable is a question worth asking. In the present paper we shall show that one of the findings which has been considered well-nigh conclusive in its support is in reality worth little as evidence. We refer to the marked siderosis of the liver parenchyma that occurs during pernicious anemia, a localization of pigment which has been taken to indicate that pathological blood destruction is localized within the portal tributaries.

Hunter [1-6] in special has laid emphasis on the difference in distribution of the hemosiderin found in the organs of cases of pernicious anemia and that laid down as the result of frank blood destruction throughout the circulation as a whole. Siderosis in instances of the latter sort is usually far more marked in the kidneys and spleen than in the liver where it is especially abundant in pernicious anemia. Hunter's contention that a portal blood destruction is thus indicated in pernicious anemia receives support from the quite different distribution of the siderosis in animals in which hemolysis has been experimentally induced by injections into

[1] Hunter, W., *Lancet*, 1888, ii, 556.
[2] Hunter, W., *Lancet*, 1888, ii, 608.
[3] Hunter, W., *Lancet*, 1888, ii, 654.
[4] Hunter, W., *Lancet*, 1903, i, 283.
[5] Hunter, W., *Lancet*, 1903, i, 367.
[6] Hunter, W., Pernicious anæmia: its pathology, septic origin, symptoms, diagnosis, and treatment, London, 1901.

the systemic circulation[3, 6, 7, 8] and in those given intravenous or subcutaneous injections of hemoglobin. Schurig,[9] for example, found that daily injections of the hemoglobin of the horse into the subcutaneous tissue of rabbits led to an abundant siderosis of the kidneys with but a slight one of the liver.

Recent work on the removal of hemoglobin from the plasma by the liver and kidneys has suggested to us the possibility that differences in siderosis such as those described might be produced merely by varying the amount of pigment set free into the general circulation. We shall record experiments which prove that this is the case and then discuss the theoretical considerations leading to and deriving from them.

Method.

Young rabbits, mostly of less than 1,500 gm. weight have been used for the work. The liver of old normal animals of many species contains not infrequently a few granules of hemosiderin, but in immature rabbits of the size indicated they are not found. The hemoglobin was prepared from rabbit corpuscles by the method of Sellards and Minot.[10] It was given in concentrated watery solution of 120 to 160 per cent hemoglobin strength (Palmer) into the subcutaneous tissue of the flanks and abdomen, care being taken to select spots where there were no large vessels; and gentle massage was done to diffuse the pigment somewhat, but not greatly, since our aim was to bring about a gradual absorption throughout the 24 hours. We were able in many instances to assure ourselves that this actually happened, by direct inspection from time to time of the injection area through the translucent skin. Injections were given on 6 days out of every 7. For purposes of tabulation (Table I) the daily hemoglobin dose is expressed roughly, in terms of the total hemoglobin of the blood of each individual, as calculated from the hemoglobin percentage (Palmer), and the total blood quantity, assuming the latter to constitute 5.5 per cent of the gross body weight. There were no great differences in the hemoglobin percentages of the animals selected, so the only important variable was the weight. Many of the rabbits grew so rapidly as to necessitate frequent increases in the amount of pigment given. The injection period ranged from 13 to 102 days and the hemoglobin dose from $\frac{1}{4}$ that normally possessed by the animal to $\frac{1}{150}$ of it. The larger injections caused some loss of body weight, but in general the health of the animals was remarkably good.

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[7] Hunter, W., Severest anæmias; their infective nature, diagnosis and treatment, London, 1909.

[8] Muir, R., and Dunn, J. S., *J. Path. and Bact.*, 1914–15, xix, 417.

[9] Schurig, *Arch. exp. Path. u. Pharmakol.*, 1898, xli, 29.

[10] Sellards, A. W., and Minot, G. R., *J. Med. Research*, 1917–18, xxxvii, 161.

The principal complication developing was a hemoglobinuria from the summated effect of the injections. Haessler [11] has noted that in the rabbit hemoglobinuria follows the rapid introduction into a vein of pigment amounts greater than $\frac{1}{125}$ of that possessed by the animal. Frequently in our experience, when a considerable amount of pigment had been introduced beneath the skin all was not absorbed before the next injection, and after a number of injections absorption would be going on from several different regions at once with result that pigment appeared in the urine. When small amounts were used, only a slight brownish stain could be detected in the subcutaneous tissue after 24 hours, yet even in such instances the urine tests were often eventually positive for hemoglobin. Whether this was due to an overtasking of the abilities of the other organs removing pigment from the blood, or resulted from changed local conditions we have not inquired. Induration at the site of injection rarely occurred.

The urine of most of our rabbits was daily submitted to the guaiac test, though, as is well known, a positive reaction does not always mean hemoglobinuria in these animals, and we were but seldom able to demonstrate blood pigment with the spectroscope. That it was actually present in the urine in many of the positive instances was indicated by their time of occurrence, which was after several large injections or a considerable number of small ones.

When large amounts of hemoglobin had been given, the renal cortex proved to be snuff-colored from siderosis, but the color of the liver was never distinctly altered from the normal, and the spleen was quite unenlarged and unchanged in hue. Chloroform was the routine lethal agent. Blocks from the liver, kidney, spleen, and red bone marrow were fixed in alcohol and stained with ammonium sulfide, and in duplicate, by the combined ammonium sulfide-potassium ferrocyanide method of Nishimura. Muir and Dunn[8] advocate the use of hot hydrochloric acid in carrying out Nishimura's technique, and, confirming their results, we find that iron is much more sharply demonstrated thereby. But since practically all of the data on the distribution of hemosiderin in human beings have been obtained by the less perfect methods above mentioned, we have given most attention to their use. The criterion used in gauging the degree of siderosis was likewise that employed with most of these data, namely the appearance of the tissue under the microscope. Since only special cells and regions of each organ undergo pigmentation, a decision as to which organ contains most hemosiderin cannot be reached, unless the differences are pronounced.

RESULTS.

The distribution of hemosiderin, as ascertained by the means described, differed markedly with the amount of hemoglobin given (Table I). When a daily portion of less than $\frac{1}{50}$ of the approximate quantity of pigment in circulation was employed, practically no

[11] Haessler, H., *J. Exp. Med.*, 1922, xxxv, 515.

TABLE I.

Relative Siderosis of the Rabbit Liver and Kidneys.

Rabbit No.	Final weight.	Proportion of animal's own hemoglobin given each time.	Period covered by injections.	Observed siderosis.	Remarks.
	gm.		*days*		
1	1,750	$\frac{1}{150}$	80	None.	No tests made for hemoglobinuria.
2	1,850	$\frac{1}{120}$	80	"	No tests made for hemoglobinuria.
3	1,600	$\frac{1}{120}$	37	Very slight; more in liver than kidneys.	Guaiac test occasionally positive; spectroscopic findings negative.
4	1,800	$\frac{1}{100}$	102	None.	No tests made for hemoglobinuria.
5	2,100	$\frac{1}{100}$	68	"	No tests made for hemoglobinuria.
6	1,550	$\frac{1}{90}$	37	Much of liver; none of kidneys.	Guaiac test occasionally positive; spectroscopic findings negative.
7	1,650	$\frac{1}{80}$	72	Much of liver; a few granules in kidneys.	No tests made for hemoglobinuria.
8	1,500	$\frac{1}{75}$	37	Much of liver; some of kidneys.	Old scarring of kidneys in both instances. Guaiac test occasionally positive; spectroscopic findings negative.
9	1,650	$\frac{1}{60}$	37	Much of liver; some of kidneys.	
10	1,650	$\frac{1}{30}$	20	Marked kidney siderosis; little of liver.	No hemoglobin ever in urine.
11	1,375	$\frac{1}{30}$	19	Similar amount in both organs.	Hemoglobin demonstrated in urine with the spectroscope.
12	1,350	$\frac{1}{20}$	18	Much kidney siderosis; little of liver.	Hemoglobin found once with the spectroscope.
13	2,000	$\frac{1}{8}$	13	Very marked kidney siderosis; little of liver.	Hemoglobin often in the urine as shown with the spectroscope.
14	1,750	$\frac{1}{4}$	13	Very marked kidney siderosis; little of liver.	Hemoglobin almost regularly in the urine.

siderosis was anywhere to be found, even after the injections had been kept up for many weeks. By the use of hot hydrochloric acid a blue dot could sometimes be demonstrated within the nucleus of many cells of the convoluted tubules of the kidneys, but with the ordinary technique it was not evident and so may be dismissed from attention. After slightly larger injections, long continued, the liver parenchyma exhibited a well defined stippling with hemosiderin, which, like that of pernicious anemia, was most pronounced near the periphery of the lobules; whereas the kidneys were non-pigmented, or negligibly so. With still more hemoglobin, the differences in the organs became less noteworthy and sometimes the kidney tubules showed an equal or greater siderosis. Always when large injections had been given, resulting in hemoglobinuria after but a few days, the epithelium of the renal tubules was heavily siderosed with coarse granules or lumps, and the hepatic parenchyma was by contrast negligibly pigmented. Often in such instances an amorphous material giving the iron reaction was to be seen lying free in the glomerular capsule and the lumen of the upper tubules, a material such as Muir and Dunn[8,12] noted after intravascular hemolysis. In the spleen no special siderosis was ever found, thus bearing out Schurig's contention that this organ, while a great locus of deposit for injured cells, has no special power to retain the pigment derived from them. The same would seem to be true of the bone marrow, to judge from our specimens.

An attempt was made to repeat the work with dogs, but several obstacles intervened that proved decisive. Dog hemoglobin has a marked tendency to crystallize out of solution. We were unable to keep it for more than a few hours in concentrations greater than 55 per cent (Palmer); and to administer the necessary amounts in dilute form entailed large injections that were often ill-borne. Furthermore, the relative vascularity of the subcutaneous tissue of the dog, as compared with that of the rabbit, and the wide extension of the injected material through it, combined to result in such rapid absorption that hemoglobinuria was rendered frequent. On the other hand, when the pigment had not been spread by massage, abscesses often developed. In the dog, too, the Kupffer cells have what would seem

[12] Muir, R., and Dunn, J. S., *J. Path. and Bact.*, 1915–16, xx, 41.

to be an unique predilection for hemoglobin. In several of our animals the liver sections submitted to the ferrocyanide reaction, appeared deep blue to the unaided eye, obviously containing enormous quantities of iron, but with the microscope this iron was seen to be confined almost wholly to the Kupffer cells which showed both a granular and a diffuse coloration. The six young dogs of our experiments were given hemoglobin in amounts of from $\frac{1}{80}$ to $\frac{1}{150}$ of that possessed in the blood. All ultimately developed hemoglobinuria and were sacrificed for that reason. The findings as regards relative siderosis of the liver and kidney parenchyma were inconclusive, as might have been predicted from these facts.

The renal siderosis in both the dog and rabbit affected especially the cells of the proximal convoluted tubules and of the ascending limb of Henle's loop, as is the case in pernicious anemia, but not in hemochromatosis,[13] a disease in which there is no evidence of pathological blood destruction.

DISCUSSION.

We have shown that the constant presence in the general circulation, as distinct from the specifically portal, of a small amount of free hemoglobin leads eventually to a siderosis of the liver similar to that which has been considered so significant in pernicious anemia. When this amount is kept within certain limits, renal siderosis fails to appear or is negligible in degree. When more is given, the epithelium of the renal tubules rapidly becomes pigmented, the iron deposition far outstripping that in the liver. These are the facts. The conception which led to the experiments demonstrating them,—and which itself receives strong support from them,—will now be outlined. It is based on the differing activities of the liver and kidneys in the elimination of hemoglobin.

The liver possesses a special ability to remove free hemoglobin from the blood stream,[14] and under normal circumstances is probably the principal organ, if not the only one, that keeps the plasma free from the pigment. It may still succeed in so doing,—and in the

[13] Gaskell, J. F., Sladden, A. F., Wallis, R. L. M., Vaile, P. T., and Garrod, A. E., *Quart. J. Med.*, 1913–14, vii, 129.

[14] Dubin, H., and Pearce, R. M., *J. Exp. Med.*, 1917, xxv, 675.

doing become siderosed—when there is a persistent slight increase in the amount of hemoglobin coming to it. Its ability, though, is overtasked by any considerable frank hemolysis,—as is often evidenced by the hemoglobinuria then ensuing; and when this is the case, the pigment accumulates in greater or less quantity in the blood, to be dealt with by the kidneys according to the laws governing the fate of threshold substances. Hemoglobin is, like dextrose and many foreign dyestuffs, a threshold substance in its renal relations,[15] one which readily passes the glomerular barrier but undergoes a greater or less resorption during its passage through the tubules. One would suppose, *a priori*, that hastening this passage would favor hemoglobinuria by lessening the opportunities for resorption, just as glycosuria is favored. And, indeed, Haessler,[11] in a paper from this laboratory, has demonstrated the fact that flood diuresis causes marked hemoglobinuria, when it is induced immediately after the intravenous injection of an amount of blood pigment so small that none passes into the urine under ordinary circumstances. All this being true, it is obvious that there must be many clinical occasions when hemoglobin passes into the tubules but fails to reach the urine, owing to the completeness of its resorption. A similar resorption of foreign dyestuffs leads to their deposition in quantity in the tubule cells. Does not renal siderosis come about in a like manner? And, when there is much blood pigment to be resorbed, will not the unusual opportunities of the tubule cells to obtain it lead to a noteworthy hemosiderin deposition within them? The results of our experiments indicate that these questions are to be answered in the affirmative. The findings do not permit of a conclusion as to whether the freedom of the kidney from hemosiderin when small amounts of hemoglobin have been administered is due to a glomerular threshold for the latter substance, as distinct from the greater renal one. Not infrequently, after large injections of hemoglobin, the amorphous contents of the glomerular capsule and tubules yields a positive reaction for iron, a fact which strongly suggests that hemosiderin may circulate as such in the blood and be excreted in the urine where indeed one of us has already found it lying free in granular form.[16]

[15] Cushny, A. R., The secretion of the urine, London, New York, Bombay, Calcutta, and Madras, 1917.

[16] Rous, P., *J. Exp. Med.*, 1918, xxviii, 645.

TABLE II.

*Relative Siderosis of the Liver and Kidney in Pernicious Anemia, Determined Microscopically.**

Author and reference.	No. of cases.	Degree of siderosis.		Technique used.
		Liver.	Kidney.	
Quincke, H., *Samml. klin. Vortr.*, 1876, No. 100,797.	2	Equal in both.		
	1	Marked.	Very slight.	
Quincke, H., *Deutsch. Arch. klin. Med.*, 1877, xx, 1.	1	"	None.	Potassium ferrocyanide and hydrochloric acid.
Quincke, H., *Deutsch. Arch. klin. Med.*, 1879–80, xxv, 567.	2	"	Slight.	Ammonium sulfide.
	2	"	None.	
Mott, F. W., *Lancet*, 1889, i, 520.	1	"	"	Potassium ferrocyanide and hydrochloric acid; also ammonium sulfide.
Mott, F. W., *Lancet*, 1890, i, 287; *Practitioner*, 1890, xlv, 81.	1	"	Slight.	Potassium ferrocyanide and hydrochloric acid; also ammonium sulfide.
Griffith, J. P. C., and Burr, C., *Tr. Assn. Am. Phys.*, 1891, vi, 239.	1	"	"	Potassium ferrocyanide and hydrochloric acid; also ammonium sulfide.
		"	None.	
Hopkins, F. G., *Guy's Hosp. Rep.*, 1893–94, l, 349.	5	Equal in both.		Potassium ferrocyanide and hydrochloric acid.
Stühlen, A., *Deutsch. Arch. klin. Med.*, 1894–95, liv, 248.	1	"	"	
	3	Marked.	Slight.	
	2	Moderate.	None.	
Warthin, A. S., *Am. J. Med. Sc.*, 1902, cxxiv, 674.	4	Equal in both.		Technique not mentioned.
	4	Marked.	Less.	
Hunter, W., *Lancet*, 1903, i, 367.	1	Slight.	Moderate.	Potassium ferrocyanide and hydrochloric acid.
	1	Moderate.	Slight.	

* The reports of Hunter's many cases [4–7] do not lend themselves well to tabulation.

TABLE II—*Concluded.*

Author and reference.	No. of cases.	Degree of siderosis.		Technique used.
		Liver.	Kidney.	
Gulland, C. L., and Goodall, A., *J. Path. and Bact.*, 1905, x, 125.	3	Equal in both.		Potassium ferrocyanide and hydrochloric acid.
	5	Marked.	Moderate.	
	3	"	Slight.	
	4	Present.	None.	
	2	"		
Schneider, J. P., *J. Am. Med. Assn.*, 1920, lxxiv, 1759.	5	Equal in both.		Technique not mentioned.
	5	Marked.	Moderate.	
	2	"	None.	

The observed variations in the distribution of hemosiderin in pernicious anemia are rendered understandable by the facts just presented. In Table II a summary is given of the records of several observers on siderosis in the disease. It will be seen that while an hepatic pigmentation not infrequently exists without any siderosis of the kidney, and is usually relatively marked when the latter occurs, yet cases are not wanting in which the renal siderosis equalled that in the liver (Table II). Such individual differences in the siderosis of the disease may be due to the same cause that has yielded like findings in the animals of our experiments, *viz.* differences in the amount of free hemoglobin in the general circulation. Livers that are damaged—and the organ is assuredly damaged in pernicious anemia as a fatty change attests—not infrequently become somewhat siderosed in the absence of a pathological blood destruction;[17] and when this is present pigmentation may become extreme.[18]

Let it be granted, despite the foregoing, that blood destruction during pernicious anemia is mainly portal. Even this constitutes no reason for the belief that the disease has its seat within the viscera draining to the liver by the portal stream. Too little notice has been taken of the circumstance that red cells, damaged in many body regions and in many different ways, are alike "scrapped"

[17] Kretz, R., *Beitr. klin. Med. u. Chir.*, 1896, No. 15.

[18] Rous, P., and Oliver, J., *J. Exp. Med.*, 1918, xxviii, 629.

within the spleen, whence the liberated hemoglobin passes to the liver. Thus it is with cells damaged by a burn of the skin,[19] by a hemotoxin,[20] or specific hemolysin injected into a peripheral vein, by chemical agents such as toluylenediamine,[21] or by the principles responsible for incompatibility on transfusion. Even under normal conditions, the same local deposition of corpuscular debris goes on,[22] as it does also to a much more considerable extent during repair from secondary anemia, when the unusually frail cells put forth by the marrow are threshed to pieces whilst in circulation.[23] All this is to say that many sorts of blood destruction which are systemic in origin become portal in completion. So it may well be with the destruction that takes place during pernicious anemia.

A brief comment upon the other evidence for a portal origin of pernicious anemia will not be out of place. Stress has been laid upon the resemblance between the disease and *Bothriocephalus* anemia, and on the results of experiments whereby a blood picture not dissimilar from that of pernicious anemia has been produced through the action of hemolytic substances absorbed from the gastro-intestinal tract. There can be, of course, no doubt that substances affecting a liberation of hemoglobin within the portal vessels will cause a more distinctive liver siderosis than those active within the circulation generally. Yet the latter can suffice as we have shown. The symptoms from the alimentary tract during pernicious anemia, and the anatomical changes ultimately found therein, constitute no stronger evidence on the seat of the disease than do the nervous symptoms and changes. The most that can be said of them is that they are suggestive. But no matter how suggestive they seem in association with the other facts just mentioned, it is surely good policy to box the compass of possibilities in the consideration of so obscure a disease as pernicious anemia, rather than to look for enlightenment in a fixed direction.

[19] Askanazy, M., in Aschoff, L., Pathologische Anatomie, Jena, 4th edition, 1919, i, 69.

[20] For example, the megatheriolysin of Todd.

[21] Joannovics, G., *Z. Heilk.*, 1904, xxv, 25.

[22] Rous, P., and Robertson, O. H., *J. Exp. Med.*, 1917, xxv, 651.

[23] Robertson, O. H., and Rous, P., *J. Exp. Med.*, 1917, xxv, 665.

SUMMARY.

The selective deposition of hemosiderin in the liver parenchyma during pernicious anemia does not constitute evidence that there is a hemolytic cause for the disease located in the portal region. The repeated introduction of small amounts of free hemoglobin into the general circulation, by the subcutaneous route, leads, as we have shown, to an identical siderosis. Larger amounts of hemoglobin cause a renal pigmentation equalling or exceeding the hepatic, a fact that is in keeping with what is known of the physiology of hemoglobin excretion and of the findings in human beings after outspoken hemolysis.

THE RENAL ELIMINATION OF BILIRUBIN.

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The mode of escape through the kidneys of circulating blood and bile pigment has received but scanty attention in the past as compared with that of foreign dyestuffs. These last have been the subject of a multitude of experiments and of controversies that are not yet at an end. The reason for this is plain. The dyestuffs have been utilized for the better discovery of principles which would apply supposedly to the body pigments as well. But since these principles are still ill defined and their general application is unattested, direct studies on the renal elimination of hemoglobin and bilirubin would seem desirable—more especially since patients with these pigments in their urine continue to demand attention.

Authorities agree that the kidney is often severely injured by jaundice. Albuminuria and cylindruria (Nothnagel) appear early. Quincke[1] has comprehensively described the anatomical changes. At an early stage the cortex is diffusely stained with bilirubin. As time passes the pigment accumulates in granular form in the cells of the convoluted tubules and more markedly in those of the loop of Henle, and in the lumen of the latter many free granules may be seen, together with yellow, green, or brown casts. It is noteworthy that the glomeruli remain practically unstained. There is cloudy swelling of the tubular epithelium, with loss of the brush border, and even necrosis here and there. In Quincke's opinion, these severe changes cannot but result in a lessened renal activity, and thus may have serious consequences for the organism as a whole.

Significance of Jaundiced Cells in the Urine.

According to text-books on clinical microscopy a "bile-stained urinary sediment" is a regular accompaniment of marked jaundice. It has been tacitly assumed that the bile staining is a staining of dead

[1] Quincke, H., in Nothnagel, H., Specielle Pathologie und Therapie, Vienna, 1899, xviii, 63.

cells by the fluid in which they lie immersed, and is devoid, therefore, of clinical significance. Such is often the case. But there is another possibility, that some of the cells may be kidney cells impregnated with pigment prior to desquamation into the urine. Such cells would afford a direct index to the renal condition, like the cells containing granular hemosiderin that are to be found in the urine of patients with hemochromatosis and pernicious anemia.[2]

In the urine of icterus neonatorum, cells containing granular and crystalline bilirubin are almost regularly present, often indeed when no dissolved pigment can be demonstrated. An excellent paper on the theme is that of Cruse[3] whose technique for the microscopic Gmelin reaction we have found highly useful. No systematic observations have been reported on the urinary cells of jaundiced adults, or at least none carried out from the standpoint of kidney physiology.

We have studied the urinary sediment of many icteric human beings and of dogs with jaundice produced in several ways—by fasting, by blood destruction with toluylenediamine or a specific hemolytic serum, and by ligation of the common duct. Two types of cell staining with bilirubin have been discriminated, one consequent on sojourn of the elements in the urine, the other a direct expression of the renal condition. In the one the cells from all parts of the urinary tract are stained, whereas in the other, no matter how deep the jaundice, the leucocytes, squamous epithelium, and bladder epithelium are uncolored, whereas the cells of manifest renal origin may be deeply stained. Needless to say, both types of staining, or only that first mentioned, will be seen in urines that have stood for a long time. The specific pigmentation of the renal cells is most evident in specimens freshly voided; and some hours are required for the bilirubin to dissolve out of the more heavily impregnated cells. After long continued jaundice in man, the urinary sediment yields striking indications of the serious condition of the kidneys. Numerous cells, often disintegrating, will be observed, crowded with coarse, opaque, brown granules or irregular particles, and many such particles lie free. All give under the microscope an intense Gmelin reaction. The pigment cannot be confused with the yellowish lipoidal substances

[2] Rous, P., *J. Exp. Med.*, 1918, xxviii, 645.
[3] Cruse, P., *Arch. Kinderheilk.*, 1880, i, 353.

found in some pathological states.[4] Intracellular clumps of narrow brown needles are frequently present. The cytoplasm of the renal cells is usually stained deep yellow, though not always. The desquamated elements from the lower urinary tract are by contrast practically colorless, though lying in a dark, icteric fluid.

Special interest attaches to the findings in subicteric states and during slight and transient jaundice. Bile pigment is a threshold substance in human beings, one which readily passes into the tubules but is absorbed again in its course through them, and so rapidly that often none can be found in the urine when a considerable quantity exists in the blood. Will the cast off renal cells yield evidence of this absorption process when the plasma contains bile pigment but tests fail to disclose it in the urine? Such has not proved to be the case. In urines that fail to give the Gmelin reaction, cells stained with bilirubin are absent. Evidently there must be in human beings a special glomerular threshold for bilirubin as well as a more general, higher one for the kidney as a whole. For were this not so, the renal epithelium found in the urine should be tinted with bilirubin whenever the plasma is colored with it—and the plasma is so colored normally.

Human urine during slight or transient jaundice regularly contains renal cells tinted a diffuse yellow and yielding the Gmelin reaction, in contrast to the colorless and negatively reacting elements of the lower tract. Granular bilirubin is not seen. We are inclined to believe that Rosenbach's method of test,[5] whereby much urine is passed through a filter and this latter submitted to the Gmelin reaction owes its delicacy, in part at least, to the accumulation upon the paper of specifically stained cells.

In dogs the slightest and most transient jaundice may lead to an output of renal cells brilliantly stippled with bilirubin. The pigment occurs as small or coarse, rounded or oblong, granules of a bright mahogany-brown, scattered irregularly in the ground glass cytoplasm of large cells with a rather small, rounded nucleus. The cytoplasm itself is usually unstained save sometimes for a very distinct brownish red zone, or halo, around each granule. In kept specimens such

[4] Weicksel, J., *Deutsch. Arch. klin. Med.*, 1919, cxxx, 260.
[5] Rosenbach, O., *Centr. med. Wissensch.*, 1876, xiv, 5.

halos of dissolved bilirubin regularly develop. These and the ruddy tint of the pigment go far to differentiate the latter from hemosiderin in the absence of chemical tests. When the urine stands for 24 hours at room temperature, the brown granulation usually disappears but in the ice box it persists much longer. Severe jaundice is accompanied in both dogs and rabbits by heavily granulated cells and free particles of bilirubin in the urine, just as in the case of human beings.

It is a curious fact that many dogs with slight jaundice yield only diffusely tinted renal cells, whereas in others with no greater icterus, elements stippled with bilirubin are regularly encountered. This difference is consistently maintained over considerable periods of time. We have noted it day after day in catheterized specimens from animals possessing normal kidneys, and with "physiological icterus" induced by fasting. It is independent of the reaction of the urine or of diuresis. Quite possibly the difference is one in derivation of the cells, those from the stippled portions of the tubules failing to desquamate in some animals.

Effects of Flood Diuresis in Dogs.

The sources of damage to the kidneys during jaundice have never been precisely determined. The excretion of bile salts may be, and probably is, far more injurious than that of bile pigment, yet there is no doubt that the accumulation of the latter in the renal cortex, as in the system generally, should be avoided if possible. Diuresis has long been advocated for the purpose. We have tested out its efficacy upon dogs.

Animals were selected that during a period of several days showed no albuminuria or casts. They were kept in metabolism cages. Under ether, all of the large bile ducts were separately ligated, or the common duct was tied and cut and the neck of the gall bladder similarly obstructed to rule out any influence of this reservoir upon the course of the jaundice. Asepsis was maintained throughout, and the incision was closed in three layers. The accumulation of bilirubin in the blood, together with its output in the 24 hour urine, was carefully followed by means of the quantitative method of van der Bergh and Snapper[6] for the blood, and Hooper and Whipple's[7] modification of the Salkowski method for the urine.

[6] van der Bergh, A. A. H., and Snapper, J., *Deutsch. Arch. klin. Med.*, 1913, cx, 540.

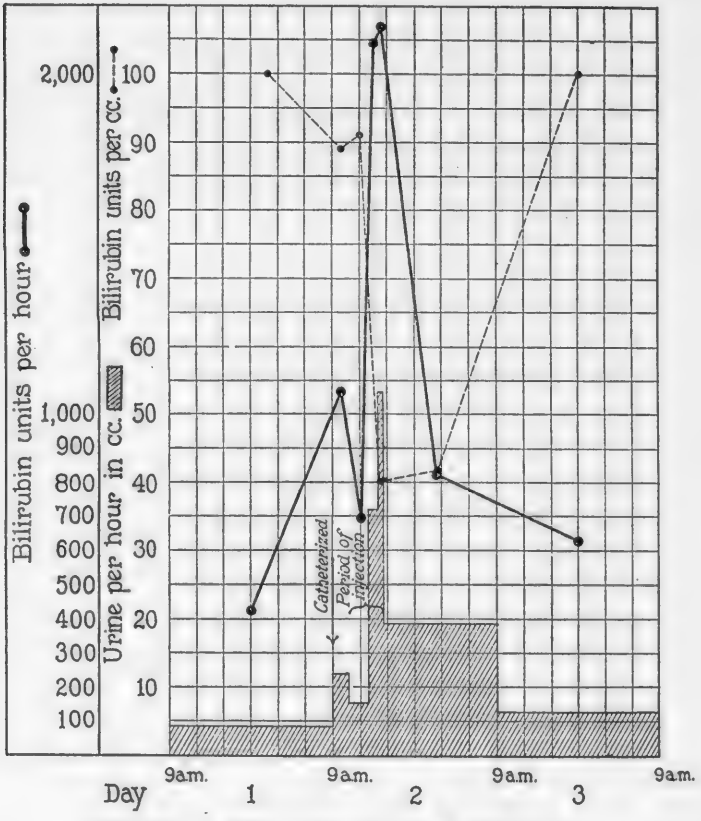
[7] Hooper, C. W., and Whipple, G. H., *Am. J. Physiol.*, 1916, xl, 332.

TABLE I.

Effect of Flood Diuresis on Bilirubinuria.

Time of occurrence after operation.	Time.	Procedure.	Fluid injected.			Urine.			Bilirubin units.			Blood.	
			Total.	Rate per kilo per min.	Period.	Total.	Output per hr.	Color.	Output per cc.	Total.	Output per hr.	Hemoglobin.	Bilirubin content per 100 cc. of plasma.
days	*a.m.*		*cc.*	*cc.*	*hrs.*	*cc.*	*cc.*					*per cent*	*mg.*
18					24	100	4.17	Dark brown.	100	10,000	417		
19	9.00	Catheterized.											
	10.00											89	1.47
	11.15	Catheterized. Injection begun.		0 37	2¼	27	12.0	" "	89	2,400	1,067		
	11.45	Speed of injection altered.	100	0.17									
	p.m.												
	2.15	Catheterized. Speed of injection altered.	325	0.185	3	23	7.7	Orange.	91	2,090	698		
	3.00		400										
	3.30	Catheterized.	500	0.37	1¼	45	36.0	Yellow.	58	2,610	2,090		
	4.00	Injection stopped.	550	0.185									
	4.15	Catheterized.			¾	40	53.3	"	40	1,600	2,133	87	1.33
	a.m.												
20	9.00				∞16¾	325	19.4	"	42	13,630	814	87	1.72
21	9.00				24	150	6.25	Light brown.	100	15,000	625		

After 10 or more days of obstruction had elapsed and the icterus had reached a relatively constant level, the study of the effects of intravenous injections of fluid was begun. The animal was stretched out; the bladder was emptied with a catheter; and warmed 0.9 per cent sodium chloride solution was introduced into



TEXT-FIG. 1. Effect of flood diuresis on bilirubinuria.

the saphenous vein at a measured rate of from 0.17 to 0.7 cc. per kilo per minute. Dastre and Loye[8] have stated that even with the maximum rate here given no fluid accumulates in the tissues or blood; and we have assured ourselves that this holds true for the blood by taking its hemoglobin content from time to time during

[8] Dastre and Loye, P., *Arch. physiol. norm. et path.*, 1888, ii, series 4, 93.

the experiment. The injection was continued for 3 hours or more, during which time the urine was collected by catheter and its pigment content determined. The experiment was successfully carried out four times in all, on three dogs.

The findings were consistent. The diuresis consequent on the injection of salt solution regularly increased the output of bile pigment greatly. The amount per cubic centimeter of urine, on the other hand, was much lessened, unlike that of hemoglobin, which under similar circumstances[9] is notably augmented. The intensity of the bilirubinemia remained practically unaffected. A single protocol will suffice to show the findings.

Experiment 1.—A brown female collie weighing 9 kilos was operated upon 19 days before the diuresis experiment and all duct branches were tied, and, where possible, cut. The amount of bilirubin contained in the urine was not measured in milligrams but in terms of an arbitrary standard of units, so called. The findings for this reason are without quantitative significance in relation to the bilirubin content of the blood. Table I gives the course and results of the experiment and Text-fig. 1 illustrates it graphically.

Effects in Dogs of Diuresis from Water by Mouth.

The effects of large amounts of water administered by mouth to dogs with the jaundice of total obstruction were followed in several series of animals.

Dogs tolerate total biliary obstruction for many weeks but lose appetite, drink little, and gradually emaciate and become anemic. The kidneys undergo a progressive injury. For these reasons it was deemed best to begin the observations within a few days after the ducts had been obstructed at operation, and by alternating periods of forced fluid by mouth with those in which there was only the normal intake to render each animal its own control. Dogs with normal blood and urine were selected for the work. Since the character and amount of the food are supposed to influence the quantity of bile pigment formed[10] a constant ration of bread and meat was supplied each day and the amount taken was determined. Fortunately the animals ate about as much when water was forced as during the control periods.

Much difficulty was experienced in determining the precise amount of pigment eliminated in the urine from day to day, more especially during the periods of diuresis. The following modification of Hooper and Whipple's method was

[9] Haessler, H., *J. Exp. Med.*, 1921, xxxv, 515.

[10] Whipple, G. H., and Hooper, C. W., *Am. J. Physiol.*, 1916, xl, 349.

finally employed. 2 per cent of the 24 hour specimen was made up to 20 cc. with water, alkalinized with sodium carbonate, the bilirubin precipitated out with calcium chloride as usual, and the bluish green coloration obtained with acid alcohol was read in a colorimeter against the color got through the action of the same kind of alcohol on a chloroform solution of bilirubin (Schuchardt) containing 1 mg. in every 4 cc.[11]

The early results indicated that not only was the bilirubin output not increased during several successive days of diuresis, but that toward the end of the period it fell almost to zero. Since such a finding is contrary to all that is known of the effects of diuresis on renal elimination, control tests were undertaken.

Various mixtures of icteric and non-icteric urines, some of them concentrated, some dilute as the result of diuresis, showed that the difficulty did not lie with the method itself, which quantitated bilirubin equally well, no matter how dilute the urine.

Further controls, involving variations in the amount of sodium carbonate used in the alkalization of the urine, and in the concentration of acid in the acid alcohol used to redissolve the precipitate, proved that these factors had no essential influence to cause error. Incidentally, it may be remarked that precipitated calcium bilirubinate was found to keep well in the ice box, and the precipitate from our specimens was sometimes kept for 24 to 48 hours prior to quantitation.

At length, tests were made to determine the value of cage as compared with catheterized specimens. The observations indicating a decreased bilirubin output during diuresis had been made upon urine collected in a vessel placed beneath the metabolism cage in which the animal was kept. Such urine was only protected from fecal contamination by a coarse grating in the bottom of the cage. The animal during non-diuresis periods drank very little water and almost invariably passed a dry formed stool, so that during these periods the amount of fecal contamination of the urine was slight. During periods of forced diuresis, on the other hand, a loose watery stool was frequently passed, and the urine specimens then contained large amounts of fecal material. Our practice had been to filter the mixed total urine for the 24 hour period and make tests on the filtered specimen. Parallel tests now made on filtered and unfiltered specimens disclosed considerable differences in bilirubin content (Table II). What is more, it was found that the sediment of a centrifuged cage specimen, though twice washed with distilled water, still may contain as much as four times the amount of bilirubin present in the urine from which it had been separated.

Since the animals serving as our example (Table II) had complete biliary obstruction, as subsequently determined at autopsy, while, furthermore, repeated

[11] McMaster, P. D., and Rous, P., *J. Exp. Med.*, 1921, xxxiii, 731.

tests of their stools when unexposed to urine invariably gave negative reactions for bilirubin, the fecal matter can be excluded as the original source of the pigment.

We believe that these findings point to a precipitation of bilirubin out of the urine by some of the fecal elements. They have been set forth at length because it is the accepted practice to use cage urine for bilirubin estimations.

TABLE II.

Effect of Fecal Contamination on the Quantitation of Bilirubin in the Urine.

Dog.	Urine specimen.	Total 24 hr. output of bilirubin as calculated from the filtered and unfiltered specimens.		Remarks.
		Filtered.	Unfiltered.	
		mg.	*mg.*	
	1	19	18	Practically no feces present.
	2	13	13	" " " "
1	3	8	23	Marked fecal contamination.
Male mongrel; weight	4	6	14	" " "
5 kg.	5	11	19	" " "
	6	11	16	" " "
	7	6	13	" " "
2	1	9	13	Marked fecal contamination.
Male mongrel; weight	2	4	5	" " "
6 kg.				
3	1	5	6	Slight fecal contamination.
Male mongrel; weight	2	9	8	" " "
8 kg.				

It has been usually assumed that in the intestinal canal bilirubin is transformed to urobilin and related substances by the action of bacteria. We have observed that when bile-containing urine is mixed with bile-free fecal matter and incubated at 37°C. for 24 hours, a large proportion of its bilirubin is destroyed, while the remainder, no longer in solution, is to be found with the fecal sediment.

Following the recognition of these sources of error, some further carefully controlled experiments were made upon the effects of diuresis in jaundiced dogs.

TABLE III.

Effect on Bilirubin Excretion of Diuresis from Water by Mouth.

Period.	Day.	Duration of excretion.	Urine.					Blood.		Water by gavage.	Remarks.
			Amount.	Output per hr.	Type of specimen.	Bilirubin content.	Bilirubin output per hr.	Hemoglobin.	Bilirubin content per 100 cc. of plasma.		
		hrs.	*cc.*	*cc.*		*mg.*	*mg.*	*per cent*	*mg.*	*cc.*	
First non-diuresis period.	1	6	72	12.0	Catheterized.	5.61	0.93	69	4.04		
		18	115	6.4	" and voided.	16.56	0.92				
		Total or average.	187	7.8		22.17	0.92				
	2	6	35	5.8	Catheterized.	7.10	1.18	69	4.06		Weight 5.2 kg.
		18	91	5.0	" and voided.	21.20	1.18				
		Total or average.	126	5.2		28.30	1.18				
	3	6	42	7.0	Catheterized.	5.41	0.90	72	4.92		
		18	109	6.1	" and voided.	17.36	0.96				
		Total or average.	151	6.3		22.77	0.95				
	4	6	24	4.0	Catheterized.	4.83	0.80	72	4.78		
		18	70	3.9	" and voided.	15.80	0.88				
		Total or average.	94	3.9		20.63	0.86				

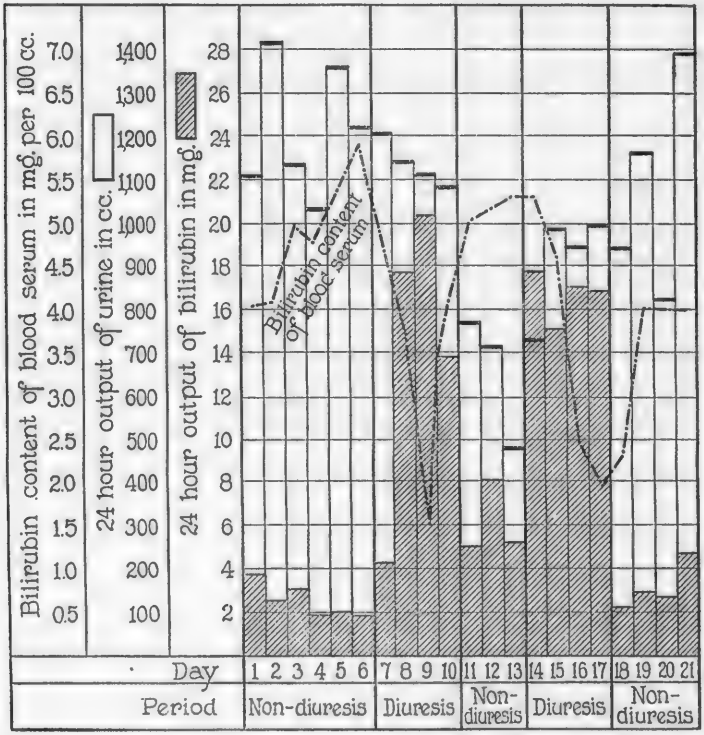
	5	24	100	4.2	Voided.	27.20	1.13				
	6	6	25	4.2	Catheterized.	5.00	0.83	72	5.90		
		18	67	3.7	" and voided.	19.40	1.08				
		Total or average.	92	3.8		24.40	1.02				
First diuresis period.	7	6	33	5.5	Catheterized.	6.43	1.07	66		500	
		18	178	9.9	" and voided.	17.71	0.99				
		Total or average.	211	8.8		24.14	1.00				
	8	6	750	125.0	Catheterized.	7.73	1.29	59	3.7	750	
		18	133	7.4	" and voided.	15.11	0.84				
		Total or average.	883	36.8		22.84	0.95				
	9	6	579	96.0	Catheterized.	7.10	1.18	72	1.5	750	
		18	439	24.4	" and voided.	15.12	0.84				
		Total or average.	1,018	42.5		22.22	0.93				
	10	6	495	82.5	Catheterized.	6.54	1.09	62	4.04	1,000	Weight 5 kg.
		18	195	10.8	" and voided.	15.22	0.85				
		Total or average.	690	28.7		21.76	0.91				

TABLE III—*Concluded.*

Period.	Day.	Duration of excretion.	Urine.					Blood.		Water by gavage.	Remarks.
			Amount.	Output per hr.	Type of specimen.	Bilirubin content.	Bilirubin output per hr.	Hemoglobin.	Bilirubin content per 100 cc. of plasma.		
		hrs.	*cc.*	*cc.*		*mg.*	*mg.*	*per cent*	*mg.*	*cc.*	
Second non-diuresis period.	11	24	250	10.4	Voided.	15.40	0.64	57	5.03		Weight 5 kg.
	12	24	405	16.9	Voided.	14.13	0.59				
	13	24	260	10.8	Voided.	9.60	0.40	56	5.30		
Second diuresis period.	14	24	885	36.8	Voided.	14.60	0.61	56	5.30	750	
	15	6	399	66.5	Catheterized.	6.27	1.04	49	4.60	750	
		18	352	19.5	" and voided.	13.50	0.75				
		Total or average.	751	31.3		19.77	0.82				
	16	6	320	53.4	Catheterized.	6.11	1.02	43	2.45	1,000	
		18	530	29.4	" and voided.	12.76	0.71				
		Total or average.	850	35.4		18.87	0.79				

	17	6	688	114.8	Catheterized.	6.11	1.02	43	1.95	800	
		18	153	8.5	" and voided.	13.79	0.77				
		Total or average.	841	35.1		19.90	0.83				
Third non-diuresis period.	18	24	109	4.5	Voided.	18.80	0.78	43	2.3		
	19	24	144	6.0	Voided.	23.20	0.97	45	4.0		
	20	24	132	5.5	Voided.	16.40	0.68				
	21	24	232	9.7	Voided	27.8	1.16	45	4.0		Weight 4.2 kg.

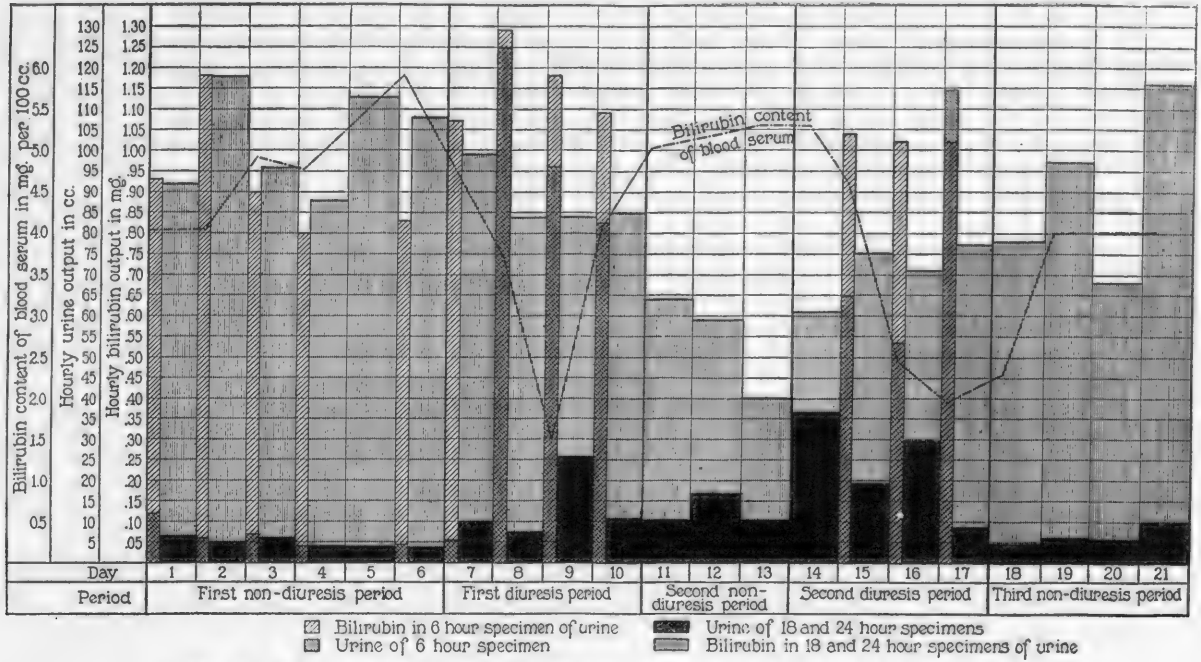
Thymol was added to the urines to check bacterial action; unfiltered specimens were employed, with their proportionate share of fecal sediment when this latter was present; and as much as possible of the 24 hour output was obtained by catheterization. Precipitation with calcium chloride was carried out on each specimen immediately after it had been procured. The bilirubin output was



TEXT-FIG. 2. Effect on bilirubin excretion in the urine of diuresis from water by mouth.

quantitated during alternate periods in one of which no effort was made to increase the water intake, which was invariably low, while in the other water was given by stomach tube, usually in 250 cc. amounts at 2 hour intervals three times each day.

A type instance of the results of the experiments is recorded in Table III and portrayed in Text-figs. 2 and 3. It will be noted that


Bilirubin content of blood serum in mg. per 100 cc.
Hourly urine output in cc.
Hourly bilirubin output in mg.
Bilirubin content of blood serum
Day
Period
First non-diuresis period
First diuresis period
Second non-diuresis period
Second diuresis period
Third non-diuresis period
Bilirubin in 6 hour specimen of urine
Urine of 6 hour specimen
Urine of 18 and 24 hour specimens
Bilirubin in 18 and 24 hour specimens of urine

TEXT-FIG. 3. The same as Text-fig. 2 except that the results are presented in greater detail.

on the 1st day when water was given by stomach tube, practically no diuresis ensued, owing probably to the fact that the animal had drunk but little for some days previously and fluid was needed by the tissues. A similar result was also obtained in later observations on diuresis in jaundiced human beings. On all subsequent days in which water was given there occurred marked diuresis, but as the greater part of the water was eliminated within 2 hours after each administration of it, there was only about 6 hours of diuresis altogether in each 24. The average urinary output for the remaining 18 hours was but slightly greater than that during the control, non-diuresis, periods. Cushny[12] has brought evidence that flood diuresis, such as follows upon intravenous injections, is practically never caused by fluid absorbed from the gastrointestinal canal, and we must assume that it had not occurred in our animals, despite the rapidity of the fluid elimination.

It will be seen that the bilirubin output per hour is fairly regular throughout each day of the first or non-diuresis period, the hourly average between 10 a.m. and 4 p.m. being in general somewhat lower than during the remaining 18 hours. Unfortunately no figures are available for the hourly output between 10 a.m. and 4 p.m. in the second and third non-diuresis periods. Only the average output per hour for the 24 hour period was recorded. In both of the diuresis periods, as already remarked, the increase in quantity of the urine was limited practically to the period between 10 a.m. and 4 p.m. The hourly output of bilirubin during this time is distinctly greater than during the remainder of the day. On the other hand, the output in the remaining 18 hours, when the urine was relatively scanty, is decreased to such extent that the output for the entire 24 hours averaged a little less than that during the first and third non-diuresis periods. True, the average 24 hour output for the second non-diuresis period is the lowest of the series, but there is no evidence to show that this was due to the administration of water during the preceding days. The main point sought after is clearly shown; diuresis from water by mouth has remarkably little effect on the day to day elimination of bilirubin.

[12] Cushny, A. R., The secretion of the urine, London, New York, Bombay, Calcutta, and Madras, 1917, 136.

The bilirubin of the blood plasma was followed by means of the diazo reaction, as has already been stated. There occurred a fall in the pigment concentration during the diuresis periods, but this can be explained in several ways other than by increased elimination of bilirubin from the body, notably by the accumulation of fluid in the tissues. Both in dogs and in human beings a fluid retention was indicated by changes in the weight. Quite possibly less bile pigment was produced in the organism during the periods of diuresis than in the intervals between.

Effects of Diuresis in Jaundiced Patients.

Observations were made on two men with catarrhal jaundice in whom periods of diuresis from forced fluid by mouth were alternated with periods of restricted water intake.[13] The icterus was marked when the observations were begun, but thereafter gradually and regularly diminished. The marked variations that were induced in the daily output of urine through forcing water by mouth had no evident effect on the rate at which the jaundice lessened from day to day as determined by the diazo reaction on the blood plasma. And the content of the 24 hour urine in bilirubin was no greater when the voidings amounted to several liters than when only half of this quantity had been passed. The findings, then, confirming those in dogs, show that diuresis by alimentary means fails, practically speaking, to increase bilirubin elimination.

DISCUSSION.

The foregoing experiments offer little support for the view advanced by some clinicians that forcing water by mouth has a direct effect to diminish the intensity of jaundice, by increasing the elimination of bilirubin in the urine. The flood diuresis which follows an intravenous injection of salt solution brings out, it is true, a relatively considerable quantity of bile pigment, but the method is clinically inapplicable, and, as has been shown, a copious diuresis from water, by mouth, yields no such consequence, so far as the 24 hour output is concerned. The fact is well recognized that "all the constituents

[13] These observations were made through the courtesy of Dr. E. F. Du Bois.

of the urine are increased in absolute amount per unit of time during diuresis."[12] In the present instance, the increase is more important theoretically than actually. Yet obviously this need not mean that diuresis is valueless as a means wherewith to combat the effects of bile retention. Its influence on the output of bile salts—substances more injurious to the organism than the pigments—remains to be determined when proper methods become available. Furthermore, diuresis may help to avert the accumulation of bilirubin in the kidneys with the disordered function consequent thereon.

Nonnenbruch[14] has shown that the acutely disordered kidney may fail to eliminate bile pigment during jaundice. It is probable that the extraordinarily pronounced icterus seen in some human beings during the later weeks of total biliary obstruction is due in part at least to a lessening of renal elimination. We have obtained data which would seem to bear significantly upon this point. In the attempt to increase jaundice in dogs, some injections of hemoglobin were given intravenously to animals already the subject of a long standing, total biliary obstruction and the mild general icterus that this entails. Hemoglobin for the purpose was obtained by the method of Sellards and Minot;[15] and eight to ten successive hourly injections were given, of amounts slightly less than that which should, on calculation, cause hemoglobinuria. In this way it proved possible to intensify the icterus markedly. At the end of a day of injections, the scleras of the dogs were pronouncedly more yellow than at the beginning. But the icterus was not maintained. By next morning only the previous, relatively pale, coloration characteristic of total obstruction was present. The urine during the transition period was heavily loaded with bilirubin. It is probable that in the case of bilirubin, as of urea, any increase in the circulating amount beyond a certain point is compensated for, when the kidneys are normal, by an increase in ease of elimination.

In man, as already stated, bilirubin is a threshold substance, in its renal relations, and it is normally present in appreciable amount in the blood. But in the dog, not only is the blood normally free from it, but whenever bilirubinemia can be detected there is bilirubinuria,

[14] Nonnenbruch, W., *Mitt. Grenzgeb. Med. u. Chir.*, 1918–19, xxxi, 470.
[15] Sellards, A. W., and Minot, G. R., *J. Med. Research*, 1917–18, xxxvii, 161.

while often the latter is to be found alone.[11] The question arises whether actually there is no threshold for bilirubin in this species, or whether the current tests for bilirubinemia are at fault. The presence in freshly voided urine, during even the slightest bilirubinuria, of renal cells specifically stained or stippled with the bile pigment constitutes evidence in this connection. According to the modern view enunciated by Cushny, all substances that pass into the urine leave the circulation by way of the glomerulus, and the so called threshold substances undergo resorption to a greater or less degree during their passage through the tubules. Thus it is that when the threshold substance is a dyestuff, the tubular epithelium becomes pigmented. Were all this quite certain, the existence in the urine of the dog of jaundiced kidney cells would be proof that in

TABLE IV.

Maximal Bilirubin Output during Total Obstruction.

Dog No.	Body weight.	Bilirubin.		Time of occurrence after operation.
		Expected 24 hr. output.	Greatest actual 24 hr. output.	
	kg.	*mg.*	*mg.*	*days*
1	4½	39.6	19.5	28
2	5⅓	45.8	28.3	28
3	9¼	81.4	24.0	10

this species bile pigment is a threshold substance. But as happens, there is recent work to show that some, at least, of the tubules have an excretory function.[16] Therefore, our cellular evidence is unconvincing.

The amount of bile pigment eliminated in the urine of the dog during complete and long continued biliary obstruction is never nearly so great as that formed during the same period by a normal liver. The bilirubin output of fistula animals in good condition amounts to about 8.8 mg. per kilo of body weight per day.[7] Table IV shows the observed pigment content of the urine of three dogs with persistent total obstruction. It will be seen that the maximum output of Dog 3 occurred on the 10th day of obstruction and that

[16] Oliver, J., *J. Exp. Med.*, 1921, xxxiii, 177.

of the other two animals on the 28th day. In all three instances the bilirubin content of the blood had already become fairly constant, and so too had the tissue icterus. The 24 hour output of pigment in the urine never approached the amount which a normal liver would have secreted in the same time. Several explanations suggest themselves for the discrepancy, more especially diminished liver function during jaundice, and destruction of pigment within the body.

SUMMARY.

The elimination of bile pigment during jaundice is, for practical purposes, unincreased by diuresis from water by mouth. Possibly, though, the flushing of the kidneys tends to lessen pigment accumulation within these organs and thus to diminish a serious potential source of trouble in long continued jaundice. Flood diuresis from intravenous injections of salt solution markedly increases the output of bile pigment. It is important to know the effect of variations in the urinary output on the elimination of bile salts, but methods for the purpose are not available at present.

The passage of bile pigment into the kidney cells during jaundice is attested by the presence in the freshly voided urine of desquamated renal elements specifically stained, stippled, or granulated with bilirubin. Pigmentation of this sort is readily to be distinguished from the indiscriminate staining of cellular debris that occurs in icteric urines on standing. It has clinical significance, furnishing direct evidence on the degree of renal change.

EXPERIMENTAL STUDIES OF THE NASOPHARYNGEAL SECRETIONS FROM INFLUENZA PATIENTS.

VII. Serological Reactions.

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Plate 37.

(Received for publication, December 10, 1921.)

During the fall and winter of 1918–19, and the early spring of 1920, we collected a number of specimens of blood serum from patients in the active stages of epidemic influenza, or after recovery from the disease, for the purpose of studying the reactions of the sera with strains of *Bacterium pneumosintes*[1] which had been isolated from the nasopharyngeal secretions of influenza patients and from the lung tissues of rabbits inoculated with these secretions. Samples of blood serum were collected also from rabbits which had been allowed to recover after showing the characteristic clinical picture produced by intratracheal injections of the active nasopharyngeal secretions, or had been experimentally inoculated with the sediment from tissue cultures of *Bacterium pneumosintes*.

The results of our efforts to demonstrate specific antibodies in these serum specimens were disappointing. The sparse growths of the organism in the earlier generations, mixed with the protein precipitate that develops in the Smith-Noguchi medium, did not provide an antigen suitable for serological tests. On account of non-specific precipitation and concomitant sedimentation, precipitin and agglutinin tests were unsatisfactory and indeterminate. Therefore, at the time the sera of influenza patients and of most of the affected rabbits were available we were unable to make use of them for lack of a suitable antigen.

[1] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 713.

More recently a method has been developed by one of us (Gates) by which certain pathogenic anaerobes, including *Bacterium pneumosintes*, may be cultivated in a collodion sac dialysate of the Smith-Noguchi medium.[2] The ascitic fluid or dilute serum and the fresh tissue fragment are placed within the sac, which is surrounded by distilled water or physiological salt solution under a vaseline seal. In the course of 24 to 48 hours anaerobic conditions are established throughout the system and the nutritive and growth-promoting substances in the tissue medium have diffused through the membrane in sufficient quantities to support growth in the surrounding liquid, while the protein precipitate that collects around the tissue fragment is retained within the sac.

Bacterium pneumosintes grows readily in this anaerobic tissue culture dialysate, visibly clouding the clear liquid in a few hours and producing a heavy turbidity in 3 to 5 days. When growth is checked through exhaustion of the nutritive material or the accumulation of deleterious substances the organisms gradually settle out of suspension, leaving a clear supernatant fluid over a compact, slightly brownish sediment. Films of these cultures show only the stained organisms, without the background of precipitate that is deposited by the tissue medium.

Preparation of Immune Serum.

When it was possible to cultivate *Bacterium pneumosintes* by this method in quantities sufficient for use, two rabbits were injected intravenously with doses of the living culture of Strain 11. This strain had been recovered from the lung tissue of a rabbit representing the eighth animal passage of active material derived from the nasopharyngeal secretions of Patient 11[3] of the 1918–19 epidemic. During immunization one rabbit (A) developed secondary infections and was killed. The other rabbit (B) received five injections of 2 to 4 cc. of a thin suspension of live culture at intervals of 5 to 7 days and was bled on the 9th day thereafter. The sterile serum was stored at 4°C. without preservative. In the first series of tests with

[2] Gates, F. L., *J. Exp. Med.*, 1922, xxxv (in press).

[3] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 125.

this serum it was examined for the presence of agglutinins, precipitins, bacteriotropins, and complement-fixing bodies against two strains of *Bacterium pneumosintes*, No. 11, the homologous strain, and No. 26, from the recurrent epidemic of 1920.

Since then the serum of this rabbit and similar sera produced in five other rabbits have been studied in various serological reactions with four strains of *Bacterium pneumosintes*, three (Nos. 11, 16, and 17) from the first epidemic (1918–19), and one (No. 26) from the second (1920). The results of the latter experiments are all in accord with those of the first series and need not be reported in detail. The following tests are described to indicate the methods and results of the first serological experiments.

Agglutination.

Agglutination tests were first made with a mixture of live organisms and undiluted immune serum from Rabbit B spread on a cover-slip, sealed with vaseline over a hollow slide, and examined microscopically immediately or after 30 minutes incubation at 37°C. Both strains tested (Nos. 11 and 26) were promptly agglutinated, with the formation of large compact masses of organisms in a clear surrounding fluid (Fig. 1). Control tests with normal rabbit serum showed no agglutination, or only the occasional clumping of three or four bacteria.

Macroscopic agglutination tests were then set up to determine the titer of the immune serum. Measured drops of serum dilutions 1:5 to 1:160 were mixed with equal quantities of bacterial suspension and sealed in capillary tubes of 2 mm. internal diameter.[4] After incubation for 2 hours at 37°C. the tubes were placed in the ice box over night and read the next morning. Strain 11 was completely agglutinated in a serum dilution of 1:80, with the limit of visible agglutination in a serum dilution of 1:320. The corresponding limits for Strain 26 were 1:40 and 1:160, respectively. Control tests with normal rabbit serum showed no agglutination even in a serum dilution of 1:2. The contrast in the behavior of Strain 11 in immune and in normal serum is shown in Fig. 2.

Precipitation.

Precipitin tests were also performed in glass tubes of 2 mm. bore, in which the antigen and the serum were carefully layered by drawing them up in succession by means of a rubber bulb. The antigen used was the clear supernatant fluid from old cultures of *Bacterium pneumosintes* grown in the dialysate of a tissue medium. After incubation for 1 hour at 37°C. a sharp opaque line of precipitation

[4] Gates, F. L., *J. Am. Med. Assn.*, 1921, lxxvii, 2054.

was observed at the juncture of the antigen and the immune serum from Rabbit B. In undiluted, normal rabbit serum a doubtful line of precipitation was observed with Strain 11 only. The appearance of the experimental and control tubes is shown in Fig. 3.

Complement Fixation.

The antigens used in the complement fixation test were the same as those for the precipitation reaction. Preliminary control tests showed that these antigens were not hemolytic or anticomplementary in volumes of 0.5 cc. when tested with 0.1 cc. of 40 per cent guinea pig complement and 2 units of anti-human amboceptor against 0.1 cc. of a 10 per cent suspension of human red cells. The immune serum of Rabbit B and the control normal serum were not hemolytic or anticomplementary in dilutions of 1:5. Antigen 0.45 cc., complement 0.1 cc., and the test sera in dilutions up to 1:160, made up to a volume of 1.05 cc. with physiological saline solution, were incubated together in a water bath at 37°C. for 30 minutes. After the addition of 0.1 cc. of corpuscle suspension and 0.1 cc. (2 units) of dilute amboceptor the tubes were again incubated for 30 minutes at 37°C. Complement fixation was complete with both antigens (Strains 11 and 26) in the highest dilution of immune serum tested; namely, 1:160. No fixation occurred in the tubes containing the normal rabbit serum.

Phagocytosis.

In the phagocytic experiments the Neufeld method[5] was used to test for the presence of bacteriotropic substances. Rabbit leucocytes were obtained from subcutaneous tubes[6] containing aleuronat in agar. Large mononuclear cells (monocytes) and lymphocytes, as well as polynuclear neutrophils, collect in these tubes and may be observed in phagocytic experiments. In the tests to be described the diluted leucocytic suspensions were mixed with suspensions of young dialysate cultures of *Bacterium pneumosintes* in physiological salt solution, or in dilutions of normal or immune rabbit serum. The mixtures were incubated 1 to 4 hours at 37°C., films prepared, fixed in methyl alcohol or by heat, and stained with Löffler's alkaline methylene blue, Wright's blood stain, or Cross' stain for leucocytes.[7] In salt solution or in normal serum controls only an occasional leucocyte picked up a few organisms. Unphagocytosed bacteria were plentiful and unagglutinated. In the presence of immune serum from Rabbit B, however, two phenomena occurred. Especially in low dilutions of the immune serum the bacteria were gathered into clumps. Leucocytes had attacked and

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[5] Neufeld, F., and Rimpau, W., *Deutsch. med. Woch.*, 1904, xxx, 1458; *Centr. Bakt., Ite Abt., Ref.*, 1906, xxxvii, 763.

[6] Gates, F. L., *Proc. Soc. Exp. Biol. and Med.*, 1920–21, xviii, 280.

[7] Cross, H. B., *Bull. Johns Hopkins Hosp.*, 1921, xxxii, 51.

sometimes surrounded these clumps, with which they were engorged. Other leucocytes had engulfed single organisms and contained from a few to very many of them. Both the polynuclear cells and the large mononuclear cells (monocytes) were actively phagocytic. Small mononuclear cells (lymphocytes) did not engulf the bacteria. The highest serum dilution in which an increased phagocytic activity was clearly evident was 1:80. Photographs of these phases of the reaction are shown in Figs. 4 to 7.

In addition to this series of experiments with the serum of Rabbit B and Strains 11 and 26 of *Bacterium pneumosintes*, we have made serological tests with Strains 16 and 17 and with the serum of five other rabbits immunized by repeated injections of these strains. Sera produced with certain strains, when tested in cross-agglutination experiments with the other strains, have agglutinated them and the homologous organisms in practically the same dilutions. No specific differences among the three strains from the 1918–19 epidemic or between any of these and Strain 26 from the recurrent epidemic of 1920 have been found, and the serological evidence indicates their antigenic identity. This is what might be expected if they were all derived from a common source.

It is evident from the results of these experiments that the reaction of the animal body to *Bacterium pneumosintes* involves the production of the antibodies which are commonly recognized by serological tests. We have no reason to suppose, therefore, that the mechanism of protection against this organism, of which we have evidence in the immunity reactions already described,[8] differs from that which comes into play in the case of infections with aerobic pathogenic organisms.

We would have welcomed the opportunity of testing the sera of influenza patients during the recent epidemic, or of rabbits experimentally infected with nasopharyngeal secretions from these patients. At first a suitable antigen was lacking. More recently almost all of the glycerolated material on hand was rendered inactive through the inadvertent use of glycerol which was later found to be chemically impure. The following single experiment, therefore, represents our only opportunity to test against *Bacterium pneumosintes* the sera of rabbits which had been subjected to the active agent in glycerolated form.

[8] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 1.

Specific Agglutinins in the Blood of Rabbits after the Injection of Glycerolated Material.

September 12, 1921. Two rabbits were injected intratracheally with glycerolated lung tissue from a previously injected rabbit which had showed the typical clinical and pathological picture already described.[3] This animal represented the seventh passage of the active agent derived from Case 26.[3] The two injected rabbits in turn showed conjunctivitis, an increase in temperature, and the characteristic fall in the total leucocyte count due to a drop in mononuclear cells. Both were allowed to recover, and were bled from the ear vein for a serum sample

		Serum dilutions											
Serum	Strain	1:2	1:4	1:8	1:16	1:32	Serum	Strain	1:2	1:4	1:8	1:16	1:32
Immune rabbit B	16	++++	++++	++++	++++	++++	Normal rabbit 3	16	−	−	−	−	−
	17	++++	++++	++++	++++	++++		17	−	−	−	−	−
	26	++++	++++	++++	++++	++++		26	−	−	−	−	−
Experimental rabbit 1	16	++++	++++	+	−	−	Normal rabbit 4	16	−	−	−	−	−
	17	++++	++++	+	−	−		17	−	−	−	−	−
	26	++++	+	−	−	−		26	−	−	−	−	−
Experimental rabbit 2	16	++++	+	−	−	−	Normal rabbit 5	16	−	−	−	−	−
	17	++++	+	+	−	−		17	−	−	−	−	−
	26	+	+	−	−	−		26	−	−	−	−	−

TEXT-FIG. 1. Agglutinins in the serum of rabbits injected with active glycerolated lung tissue.

on the 21st day after injection. These sera together with three normal rabbit sera and the immune serum from Rabbit B were then tested for agglutinins against killed, washed, dialysate cultures of *Bacterium pneumosintes* (Strains 16, 17, and 26). The results of this experiment are shown in Text-fig. 1.

This experiment shows that the animal body reacts to experimental infection with the active agent of the nasopharyngeal secretions of influenza patients by antibody formation against *Bacterium pneumosintes*, and completes the proof of the identity of the active agent with the organism obtained from the same immediate and original sources.

SUMMARY.

Cultivation of *Bacterium pneumosintes* in the collodion sac dialysate of a tissue medium produces an antigen suitable for serological tests.

Injection of dialysate cultures of *Bacterium pneumosintes* into rabbits results in the production of antibodies demonstrable by agglutination, precipitation, complement fixation, and phagocytic reactions.

Four strains of *Bacterium pneumosintes*, three from the first epidemic influenzal wave (1918–19) and one from the second (1920), show identical antigenic characters.

The blood serum of rabbits experimentally injected with the glycerolated active material of rabbit passages contains specific agglutinins for *Bacterium pneumosintes*, whereas normal rabbit serum does not.

EXPLANATION OF PLATE 37.

FIGS. 1 and 2. Agglutination of *Bacterium pneumosintes* in immune serum from Rabbit B.

FIG. 1. Edge of hanging drop, dried and stained. × 15.

FIG. 2. Macroscopic agglutination in capillary tubes. *A*, immune serum, complete agglutination; *B*, normal rabbit serum, no agglutination. The white crescent at the bottom of each column is not sediment but light reflected from the meniscus. × 1.

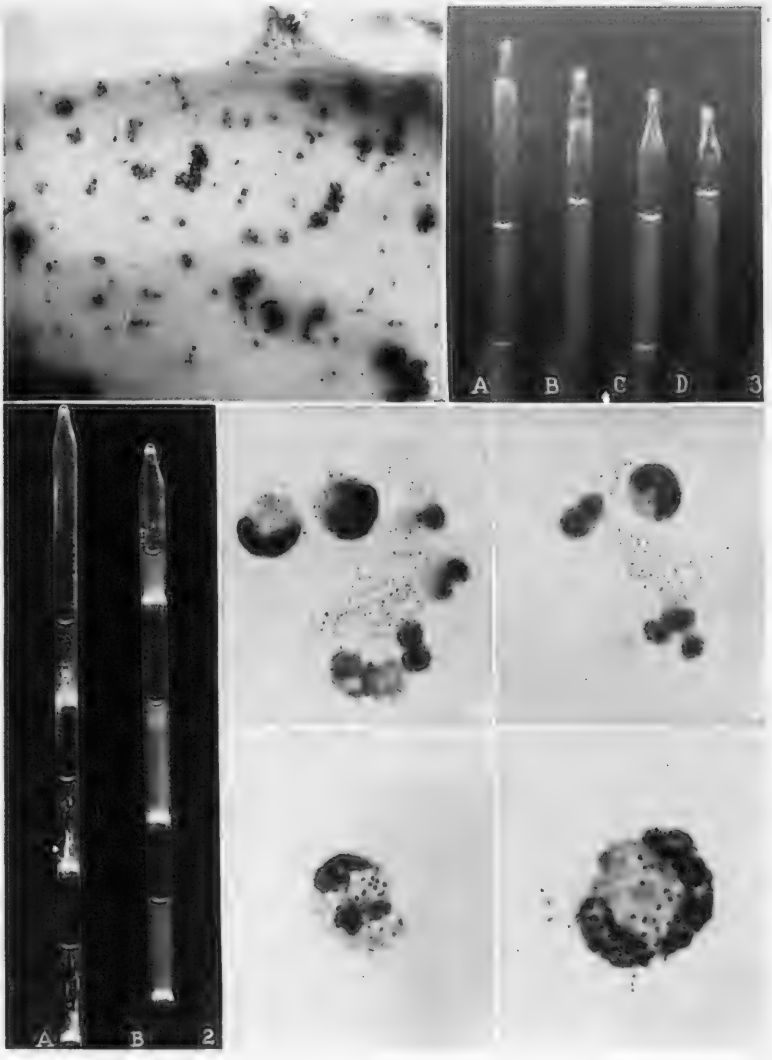
FIG. 3. Precipitation reaction with the supernatant fluid of *Bacterium pneumosintes* cultures *versus* rabbit serum. *A*, Strain 11 and immune serum from Rabbit B; *B*, Strain 11 and normal rabbit serum; *C*, Strain 26 and immune serum from Rabbit B; *D*, Strain 26 and normal rabbit serum. × 1.

FIGS. 4 to 7. Phagocytosis of *Bacterium pneumosintes* in immune serum from Rabbit B.

FIGS. 4 and 5. Monocytes (transitional cells) attacking agglutinated groups of organisms. × 1,000.

FIG. 6. Polynuclear neutrophil containing organisms. × 1,100.

FIG. 7. Monocyte containing organisms. × 1,500.



(Olitsky and Gates: Nasopharyngeal secretions from influenza. VII.)

MUTATION OF THE BACILLUS OF RABBIT SEPTICEMIA.

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Plate 38.

(Received for publication, December 7, 1921.)

INTRODUCTION.

Two varieties of microbe have been shown to exist in cultures of the rabbit septicemia bacillus (1). These organisms have been designated as Types D and G. Type D is the microbe invariably obtained at necropsy of rabbits dying from natural infection. Type G appears after artificial culture has been carried out for some time. It was important to determine whether the two varieties coexist in cultures isolated from infected rabbits, or whether Type G arises by mutation from Microbe D. Much of the work on mutation of bacteria has been criticized because of failure to employ pure-line strains, that is to say, cultures arising from single organisms. Since the primary isolations of Microbe D were made from colonies which conceivably might arise from two or more organisms, it would be unjustifiable to conclude that the original Type D had changed into the microbe of the G variety. Consequently, the experiments about to be reported were performed with pure-line strains isolated from a Type D culture by the Barber method (2).

Differential Characteristics of Microbes D and G.

These have been given in detail in a preceding communication (1) but will be briefly summarized here.

Type D is very virulent for rabbits, grows diffusely in liquid media, and yields smooth *fluorescent* colonies on serum agar. The acid agglutination optimum lies between pH 3.5 and pH 3.0 (Michaelis-Beniasch buffer series (3)).

Type G is of very low virulence for rabbits, exhibits granular growth in fluid media, and grows in the form of slightly irregular, translucent, *non-fluorescing*

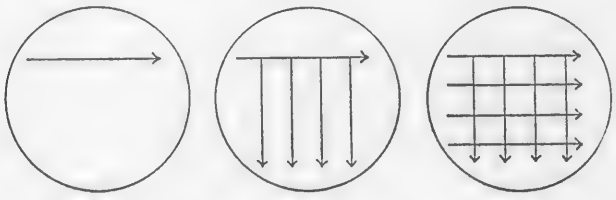
colonies on serum agar. Its acid agglutination optimum lies between pH 5.0 and pH 3.5.

There is no noticeable morphological difference between the two varieties. Their fermentation reactions are indistinguishable. Immunization and agglutination absorption experiments indicate that they are antigenically homologous.

Method of Detection of Microbe G in Cultures in Which Microbe D Predominates.

This is made comparatively easy by the striking difference in appearance of the colonies on streaked serum agar plates, especially when the latter are held at certain angles before artificial light.

The tube of liquid medium supposed to contain the mixture of Types D and G is thoroughly shaken in order to bring into suspension clumps of Type G which may possibly have sedimented. A large



TEXT-FIG. 1, *a* to *c*. Diagrammatic sketch of the method of streaking serum agar plates.

platinum loop of the material is then streaked over the surface of 10 per cent rabbit serum agar in Petri dishes of 15 cm. diameter.

The loop is then sterilized, and successive streaks are made at right angles to the first one and starting from it. Then, without sterilizing the loop, it is passed across these second streaks and parallel to the original one. The resulting growth will therefore have a checkerboard appearance. The growth is heavy in the upper left-hand corner, the point where the loop was first applied. It will be much scantier in the lower right-hand corner. The successive stages of the operation are shown in the diagrammatic sketches in Text-fig. 1, *a* to *c*.

The result may be seen in Fig. 1. In this figure the Type D colonies can be readily detected, though in the minority. They are white while the Type G colonies appear gray.

The comparative scantiness of the colonies in the lower latitudinal streaks makes it possible to make rough quantitative estimates of the relative proportion of the Type D and G colonies.

Isolation of Pure-Line Strains.

The rabbit septicemia bacillus is rather exacting in its nutritive requirements, and it is necessary to make fairly generous seedings during routine transplant. As a result, difficulty was at first experienced in obtaining positive cultures by the Barber method. This was overcome by performing the entire operation in undiluted rabbit serum. The cultures used for fishing had been incubated in undiluted rabbit serum for 3 hours. Minute drops of these young cultures were placed in the usual manner in the Barber cell, examined by oil immersion lens, and when found to contain single organisms, fished to undiluted rabbit serum, and incubated. The percentage of positive cultures obtained by this method is relatively high. On the other hand, in plain or serum broth the results are very unsatisfactory.

EXPERIMENTAL.

The following observation led to the experiments about to be presented. Several strains of Microbe D, isolated at necropsy from rabbits dead of bronchopneumonia, were carefully purified as follows: A 6 hour serum broth culture was diluted in broth to 10^{-8} cc. of the original. Platcs in serum agar made from this dilution usually showed from two to five colonies. One of these was fished to serum broth, and as soon as a cloud appeared (about 6 hours) the dilutions were again made and 10^{-8} cc. plated as before. This process was repeated four times in all. The young cultures used for these dilutions showed few or no clumps. The high dilutions—10^{-8}, 10^{-7}, and 10^{-6} cc.—when plated, yielded numbers of colonies that increased in direct proportion with the lowering of the dilution. That is to say, 10^{-8} plate, 2 colonies; 10^{-7} plate, 19 colonies; 10^{-6} plate, 205 colonies. These considerations make it certain that at some, and probably at each time during the fishing process a colony was obtained arising from *one* organism.

The Type D strains so purified were now subcultured daily in undiluted rabbit serum and in plain broth. Each culture was streaked

on 10 per cent rabbit serum agar in the manner just described. Careful inspection for Type G colonies was made. From the twentieth to the twenty-fifth passage, these began to make their appearance in small numbers in the plain broth series. None could be demonstrated in the plates made from undiluted serum.

One tube of the plain broth series which had shown *no* Type G colonies was allowed inadvertently to stand for 4 days at room temperature. It was then streaked, and large numbers of Type G forms appeared on the serum agar plate. None were present in a 4 day serum culture of the same Type D strain. The Type G colonies, subcultured from the plain broth plate, bred true to type and showed all of the characteristics previously described. It was concluded that an undoubted mutation of the original Type D strain had occurred. Eight pure-line strains were then obtained by the Barber method and the following experiments made, to determine, if possible, the conditions favoring and inhibiting the D → G transformation.

Experiment 1. D → G Mutation in Plain Broth, Serum Broth, and Undiluted Rabbit Serum.—0.05 cc. of a 6 hour undiluted serum culture of Microbe D, pure-line, was inoculated into three large test-tubes containing 10 cc. respectively of undiluted rabbit serum, 5 per cent rabbit serum broth, and plain broth. The tubes were placed in the incubator at 37°C., and streaked on serum agar plates by the method just described, at intervals covering a period of 109 hours. The plates were incubated for 24 hours and then inspected for the appearance of Type G colonies. The serum culture used for inoculation was streaked and observed to contain only Type D. The result of the experiment is given in Table I.

The result of Experiment 1 indicates that the ageing of a culture plain broth causes Microbe D to change into Type G. The addition of a small amount of serum does not greatly inhibit this change. On the other hand, undiluted rabbit serum has a very marked inhibitory effect.

All of the pure-line strains under study have been found to undergo this mutation when allowed to age in plain broth, but do so with varying rapidity and completeness. The Type G colonies, fished into plain broth or undiluted serum, remained true to type indefinitely. They showed no tendency to revert to the parent D form even when transplanted continuously in undiluted rabbit serum, a medium which is antagonistic to the original change.

It was thought that it might be possible to demonstrate mutation-enhancing properties in plain broth cultures of Microbe D. The following experiment examines this question.

Experiment 2. Effect of Type D Culture Filtrates on the Mutation Rate of Microbe D.—A pure-line strain of Microbe D was planted into a series of flasks containing plain broth (pH 7.4). These were incubated at 37°C. After 6, 24, 48, and 72 hours, a flask was removed, centrifuged, and filtered through small Berkefeld candles. The filtrates upon test proved to be sterile. A slight change in reaction had occurred, the 6 and 24 hour filtrates giving pH 7.1, the 48 hour pH 7.0, and the 72 hour pH 6.9. The filtrates were now divided into two

TABLE I.

D → G Mutation in Plain Broth, Serum Broth, and Undiluted Rabbit Serum.

Strain used for inoculation.	Medium.	Proportion of Types D and G.							
		9 hrs.	24 hrs.	36 hrs.	48 hrs.	60 hrs.	72 hrs.	85 hrs.	109 hrs
	Undiluted rabbit serum.	D	D	D	D	D	D	D	D
0.05 cc. of 6 hr. serum culture; pure-line strain; Type D.	5 per cent serum broth.	"	"	"	"	"	" +2G.	" + several G.	" + G (about 70:30).
	Plain broth.	"	"	"	" + few G.	" + few G.	D + moderate number of G.	D + many G.	D + G (about 50:50).

parts, half was titrated back to pH 7.4 with 0.1 N NaOH, the remainder was left without treatment. 10 cc. of each filtrate were placed in large test-tubes. These tubes were now seeded with 0.05 cc. of serum culture of pure-line strain, Type D. Controls, consisting of similar amounts of sterile broth incubated for 6 and for 72 hours, and of undiluted rabbit serum, were similarly seeded. All of the tubes were now incubated at 37°C., and streaks made on serum agar plates at intervals up to 7 days, 10 hours. Growth occurred in all of the filtrates, although a considerable lag period was evident in those of 48 and 72 hours. The results of the experiment are presented in Table II.

TABLE II.

Effect of Type D Filtrates on the Mutation Rate of Microbe D.

Tube No.	Medium.	pH	Proportion of Types D and G.									
			12 hrs.	28 hrs.	43 hrs.	60 hrs.	72 hrs.	84 hrs.	106 hrs.	120 hrs.	138 hrs.	176 hrs.
1	6 hr. filtrate.	7.1	D	D	D	D	D	D + 1 G.	D	D	D	D
2	6 " "	7.4	"	"	"	"	"	D	"	D + 2 G.	D + few G.	"
3	24 " "	7.1	"	"	"	"	" +2 G	" + 1 G.	"	" + few G.	D	D + few G.
4	24 " "	7.4	"	"	"	"	D	D	"	" + " "	"	D
5	48 " "	7.0	"	"	"	"	"	"	D + 1 G.	D + moderate number of G.	D + few G.	D + few G.
6	48 " "	7.4	"	"	"	"	"	"	D	D + moderate number of G.	" + " "	" + " "
7	72 " "	6.9	"	"	"	"	"	" + 1 G.	D + moderate number of G.	D + G (50:50).	D + G (50:50).	D + G (50:50).
8	72 " "	7.4	"	"	"	"	"	D	D + moderate number of G.	D + G (50:50).	D + G (40:60).	D + G (50:50).
9	Sterile broth (37°C. 6 hrs.).	7.4	"	"	"	"	"	" + 12 G.	D + many G.	D + G (40:60).	D + G (40:60).	D + G (40:60).
10	Sterile broth (37°C. 72 hrs.).	7.4	"	"	"	"	"	D + 6 G.	" + " "	D + G (30:70).	D + G (30:70).	D + G (50:50).
11	Undiluted serum.	7.4	"	"	"	"	"	D	D	D	D	D

A glance at Table II shows that the expectation of the discovery of conditions enhancing mutation was not verified. In fact, the opposite property is seen to be present in the 6, 24, and 48 hour filtrates. Contrary to expectation, the number of Type G colonies arising in the 6 and 24 hour filtrates was extremely small, and comparatively few appeared in that of 48 hours. In the 72 hour filtrate the mutation rate paralleled that of the control broth. The mutation had reached 50 per cent or more in 176 hours. In undiluted rabbit serum no Type G colonies appeared at any time during the experiment. Early filtrates from Type D cultures exhibit a markedly antagonistic action to the mutation. Little or no difference is to be observed between the 72 hour filtrate of reaction pH 6.9 and its companion tube, which had been titrated back to pH 7.4. It would appear that the slight change in reaction has no effect upon the mutation. Many of the Type G colonies were subjected to the differential tests, and proved in all cases to be authentic Type G cultures. They did not revert to Type D.

An effort was now made to discover, if possible, the constituents of plain broth that encourage the D → G mutation. Plain beef infusion was the first of these elements subjected to test. The beef infusion was prepared in exactly the same way as in the preparation of plain broth. It was titrated to pH 7.4. The only difference between it and the plain broth ordinarily used was the absence of peptone (Fairchild's) and of Na_2HPO_4, which is added for buffer purposes.

Experiment 3. D → G Mutation in Beef Infusion and Other Media.—0.05 cc. of a serum culture of a pure-line strain, Type D, was seeded into large test-tubes containing respectively 10 cc. of 6 hour Type D filtrate, of plain broth, of 5 per cent rabbit serum broth, of beef infusion, of 5 per cent rabbit serum-beef infusion, and of undiluted rabbit serum. The material used for seeding was streaked on serum agar plates and proved to contain only Type D microbes. The plain broth was made up from the beef infusion used in the experiment. All of the tubes were placed at 37°C. in the incubator and tested by streaking on serum agar plates at intervals up to 18 days. The results are given in Table III.

Table III indicates that beef infusion is very unsuitable to the D → G change. In 228 hours, frequent streaking of 10 per cent serum agar plates revealed only one Type G colony in this medium. In 18 days

a considerable mutation had taken place. The process, therefore, can take place in this medium, but is undoubtedly greatly retarded in comparison with the mutation occurring in plain broth. It is interesting to note that in the beef infusion-serum mixture, only one Type G

TABLE III.

D → G Mutation in Beef Infusion and Other Media.

Period of incubation at 37°C.	Proportion of Types D and G.					
	Tube No.					
	1	2	3	4	5	6
	6 hr. Type D filtrate.	Plain broth.	5 per cent serum broth.	Beef infusion.	5 per cent serum-beef infusion.	Undiluted serum.
hrs.						
12	D	D	D	D	D	D
24	"	"	"	"	"	"
36	"	"	"	"	"	"
52	"	D + few G.	"	"	"	"
84	"	" + " "	"	"	"	"
96	D + 3 G.	D + moderate number of G.	D + 1 G.	"	"	"
120	" + few G.	D + many G.	D	"	"	"
144	" + few G.	D + G (70:30).	"	D + 1 G.	"	"
173	" + few G.	D + G (30:70).	D + many G.	D	"	"
197	D	D + G (30:70).	D + many G.	"	D + 1 G.	"
228	"	D + G (40:60).	D	"	D	"
days						
18	"	D + moderate number of G.	"	D + G (95:5).	"	D + few G.

colony was observed over a period of 18 days. The 6 hour Type D filtrate showed its usual inhibitory activity. This was the same filtrate as that used in Experiment 2. It had been kept in the ice box for 9 days after that experiment. In undiluted rabbit serum no Type G colonies were observed for 228 hours. A few were revealed by the examination after 18 days. This indicates, again, that the process

D → G takes place in a variety of media, but that the tendency is far more marked in some than in others.

A further interesting fact is to be observed in Table III. It will be noted that in Tube 2 (plain broth) the proportional number of Type G colonies reaches a maximum after 173 to 197 hours, is less in 228 hours, and far less after 18 days. This is also true of the serum broth (Tube 3). This phenomenon has been observed many times and its analysis is demanded. The great decrease in the relative number of Type G colonies must be due to one of two causes. Either the mutant G forms die off more rapidly than do the parent D microbes, or an equilibrium is established in the tubes which results in a tendency of the newly mutated Type G to revert to the original Type D. Such reversion has never been noted in subcultures made by fishing Type G colonies. On the other hand, it is conceivable that an equilibrium might be set up in the original reaction tube.

TABLE IV.

D → G Mutation in 2 Per Cent Peptone (Fairchild's).

Period of incubation at 37°C., *hrs.*	15	30	48	72	96	134	146	183
Proportion of Types D and G.	D	D + 3 G.	D	D	D + G (40:60).	D + few G.	D	D

The striking retardation of D → G mutation in beef infusion led to the examination of peptone as a possible factor in the hastening of the process. Preliminary experiments indicated that it was necessary in order to obtain growth to seed relatively large amounts of Microbe D into the peptone solutions.

Experiment 4. D → G Mutation in 2 Per Cent Peptone (Fairchild's).—The peptone solution was made up in distilled water and titrated to reaction pH 7.4. A large test-tube containing 10 cc. of this medium was then seeded with 0.5 cc. of a 10 hour culture of pure-line strain, Type D, in peptone. The tube was incubated at 37°C. for 183 hours and tests on serum agar plates were made at intervals dusing this time. The result is given in Table IV.

Marked mutation is seen to occur in peptone solution, and the phenomenon of a decrease in the number of Type G colonies after a maximum is strikingly manifest in this experiment.

TABLE V.

D → G Mutation in Various Concentrations of Peptone (Fairchild's).

Tube No.	Medium.			Proportion of Types D and G.						
	Peptone 20 per cent.	Beef infusion.	Final per cent of peptone.	24 hrs.	48 hrs.	72 hrs.	96 hrs.	120 hrs.	144 hrs.	192 hrs.
	cc.	*cc.*								
1	10.0		20.0	D	D + 7 G.	D + 10 G.	D + G (50:50).	G + few D.	G + 2 D.	
2	5.0	5.0	10.0	D + 3 G.	D	D + many G.	D + G (90:10).	D + G (10:90).	D + G (10:90).	D + G (50:50).
3	2.5	7.5	5.0	D	D + G (90:10).	D + G (40:60).	D + G (15:85).	D + G (2:98).	D + G (2:98).	D + G (50:50).
4	0.5	9.5	1.0	"	D	D	D	D + few G.	D + few G.	D
5	0.1	9.9	0.2	"	D + 3 G.	"	"	" + " "	D	"
6		10.0		"	D	"	"	" + 2 G.	"	"
7	Plain broth.			"	"	D + 8 G.	"	D + many G.	D + many G.	D + few G.
8	Rabbit serum undiluted.			"	D + few G.	D	D + 1 G.	D	D	D

The next experiment dealt with the effect of various concentrations of peptone on the process. A stock solution of 20 per cent Fairchild's peptone was prepared and titrated to pH 7.4.

Experiment 5. D → G Mutation in Various Concentrations of Peptone (Fairchild's).—Eight large tubes were prepared containing respectively 20 per cent peptone solution undiluted, 20 per cent peptone solution diluted to varying degrees with beef infusion, beef infusion alone, plain broth, and undiluted rabbit serum. The pH of all of these media was 7.4. They were seeded with 0.1 cc. of a pure-line strain of Microbe D, 6 hour culture in rabbit serum, which showed only Microbe D when streaked on serum agar. The tubes were then placed in the incubator at 37°C. and streaked on serum agar plates at intervals up to 8 days. The results of the experiment are given in Table V.

Table V shows in a very striking manner the marked effect of high concentration of peptone on the mutation. The process occurs much more rapidly than is usually the case in plain broth, and, what is more, in the case of 20, 10, and 5 per cent peptone, goes much nearer to completion. That is to say, the mutation instead of involving 30 to 40 per cent of the organisms, reaches 90 per cent and in some cases arrives almost at completion. On the other hand, the tubes with very low concentrations of peptone or with beef infusion alone and with undiluted rabbit serum show a very few Type G colonies during the entire period of the experiment and in the last two tests proved to contain nothing but Type D. The decrease in relative number of Type G colonies is again strikingly apparent in Tubes 2 and 3, 192 hour test.

It cannot be said that the presence of the peptone causes the mutation D → G, since the change occurs occasionally, though only to a small extent, in beef infusion and in undiluted rabbit serum. But it is certain that the presence of peptone in suitable concentrations greatly accelerates a reaction toward which a tendency already exists. It is interesting that four pure-line strains, kept on ice in undiluted rabbit serum for 3 months without passage, showed no evidence of the appearance of Type G colonies when subsequently transplanted.

Some preliminary experiments have been made in regard to the effect of varying $C_{II}+$ of the medium upon the D → G process. These of course are limited by the range within which good growth occurs; *i.e.*, about pH 8.5 to pH 6.0. The results would indicate that

reaction of pH 6.0 distinctly retards the process, while it is, if anything, accelerated in reaction pH 8.5.

DISCUSSION.

Great confusion exists among bacteriologists in regard to the meaning of the term mutation. Its use in application to the experiments just described is considered to be entirely justified in the light of the definition of Dobell (4), who speaks of it as "a permanent change—however small it may be—which takes place in a bacterium and is then transmitted to subsequent generations." The Type G colonies arising in these experiments, and subcultured to undiluted rabbit serum, were frequently tested for the characters that distinguish them from the parent Type D. The mutant G forms were found in every case to possess little or no virulence, to grow in a granular fashion in fluid media, and to possess an acid agglutination point distinctly different from that of Type D. All of these characters persist throughout many passages in undiluted serum, a medium in which the original change seldom, if ever, takes place. It would seem that if a so called atavistic reversion were to take place, it would certainly occur in undiluted serum. This has never been observed.

The majority of authentic examples of bacterial mutation has been concerned with gain or loss in fermentative power. We refer here only to work performed with pure-line strains. Among these researches, the investigations of Benecke (5) and Kowalenko (6) deserve to be mentioned. These investigators found that Massini's (7) *Bacterium coli mutabile* gained the power of splitting lactose when cultivated in this sugar and that this power persisted indefinitely. The work of Müller (8) in regard to *Bacillus typhosus* is of a similar nature as is that of Burri (9) with *Bacterium imperfectum*. It would seem that the D → G process just described involves a much more fundamental change in the bacterial cell. The distinct difference in acid agglutination optimum is a remarkably regular occurrence. It is in the nature of a physical constant for each type and would imply an important change in the protoplasm of the organism. It is certain that this phenomenon is not confined to the hemorrhagic septicemia group, of which the rabbit septicemia bacillus is a member. Varieties showing granular growth character have been discovered in

typical cultures of paratyphoid organisms by Krumwiede (10), of *Bacillus dysenteriæ* Shiga by Arkwright (11) and Zoeller (12), and in cultures of *Bacillus coli* by Gratia (13). The way in which these have arisen has not been taken up by these authors, but it is quite certain to be found of a nature closely akin to the process just described.

SUMMARY AND CONCLUSIONS.

Type G microbes, discovered in pure cultures of the rabbit septicemia bacillus, have been demonstrated to arise from the parent D form by mutation.

The D → G mutation takes place in broth cultures of pure-line strains of Microbe D, when these are kept for several days without transplant at 37°C., or at room temperature, or in the ice box.

The mutation is greatly inhibited by filtrates from 6 and 24 hour cultures of Microbe D, and to some extent by filtrates from 48 hour cultures.

The process of transformation takes place to a very slight extent or not at all in undiluted rabbit serum, but Type G colonies subcultured to this medium do not revert to the parent D form.

The D → G change is strongly inhibited in cultures made in simple beef infusion, or in 5 per cent rabbit serum-beef infusion.

Peptone would seem to be the constituent of plain broth which favors the process. In high concentrations of peptone, the mutation is rapid and may reach a degree of 90 per cent of the total organisms in 5 to 6 days.

A distinct maximum of the relative number of Type G colonies as compared to the parent Type D is observable in plain broth and in some concentrations of peptone, when these are kept at 37°C. for some days without transplant. Subsequent tests show the concentration of Type G microbes to diminish.

The change in acid agglutination optimum exhibited by the mutant G forms implies a distinct change in bacterial protoplasm and would seem to be one of the most fundamental mutations so far described.

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EXPLANATION OF PLATE 38.

FIG. 1. 10 per cent rabbit serum agar plate showing mixed culture of Microbes D and G. Type G preponderates and has a grayish appearance. The Type D colonies are white. The arrows indicate type D colonies.

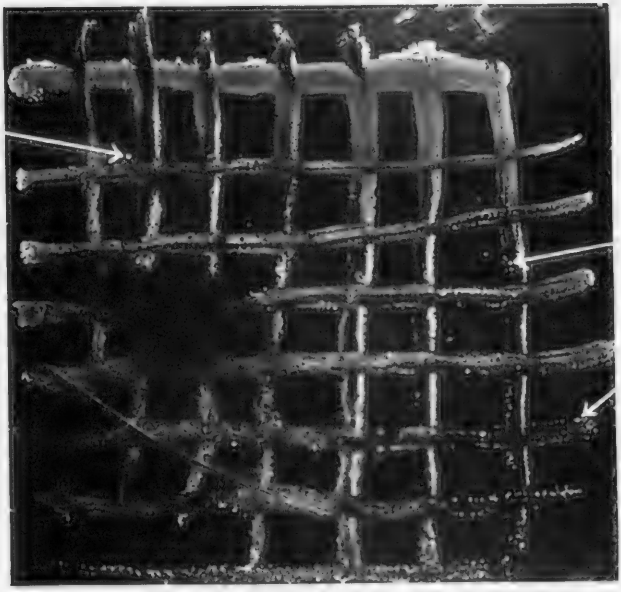

FIG. 1.

(De Kruif: Mutation of bacillus of rabbit septicemia.)

SPONTANEOUS DECREASE OF THE SURFACE TENSION OF SERUM. I.

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When a serum is exposed to the air, the surface energy of its free surface decreases progressively, according to a certain law, until after about 10 minutes a state of equilibrium is attained. The purpose of this paper is to report a study of this phenomenon, which has been overlooked so far on account of lack of proper apparatus for measuring surface tension.

The existence of a film at the surface of protein solutions has been discussed by Ramsden.[1] He pointed out the formation of semisolid or highly viscous films, and merely noted that "the matter which accumulates [in the surface layer] possesses the property of lowering the surface tension . . . . of the free surface of water."[1] The technique which he used did not allow him to proceed further in that line, and he neither observed nor measured any lowering in the surface tension of the serum itself, not did he notice the variations of the surface tension of water plus serum in function of the time. There is no doubt but that the existence of a more concentrated layer at the free surface or at all interfaces plays an important part in most biological phenomena. Whether this "film" is solid or liquid with simply higher viscosity will be discussed later. In order to prevent any misunderstanding, the word film will only be used to define a liquid film; the term "membrane" will be used with reference to a solid film.

I.

EXPERIMENTAL.

Over 3,000 measurements of surface tension were made. The instrument used was the tensiometer, previously described.[2] The

[1] Ramsden, W., *Proc. Roy. Soc. London*, 1904, lxxii, 156.

[2] du Noüy, P. L., *J. Gen. Physiol.*, 1918–19, i, 521.

use of this apparatus is based upon the adhesion of a platinum ring to the surface of the liquid, and the stress necessary to tear off the film is measured by the torsion of a steel wire. A measurement with this instrument requires approximately only 20 seconds, and the instrument always measures the tension of the same layer of liquid.

The ring method was adopted, after much experimenting, as the only reliable one for colloidal solutions, as well as the simplest, quickest, and most accurate. This opinion is now shared by a large number of physicists, and particularly by Ferguson, who reported about twenty different techniques for measuring surface tension.[3]

The tensiometer[4] was used with an accurately calibrated platinum-iridium ring (circumference 4.00 cm.), and is reliable to ± 0.1 dyne. We believe that any data giving the second and third decimals are useless in general because, so far, none of the twenty odd methods in existence check each other, some differing by more than 5 dynes for the same substance at the same temperature, and because no theoretical reasons exist yet for adopting a standard value of surface tension for any substance.

All measurements were made between 23° and 25°C., room and liquid temperature. All glasses, containers, and pipettes were boiled in cleaning solution (sulfuric acid and sodium dichromate), then rinsed in distilled water, and dried. The free surface of the liquid was always 10 sq. cm. with a tolerance of ± 0.5 sq. cm. The elapsed time between the pouring of the liquid and the measurement was very nearly constant, and equalled 15 seconds. The ring was washed in distilled water and flamed after every measurement. The same precautions were taken in collecting and preparing the serum. Only clean glass was used. No alcohol or ether was ever employed. When it occasionally happened that the edge of a tube had been touched with the fingers, a marked difference was observed in the readings.

The results are shown in Table I.

Text-figs. 1 and 2 show more clearly the progressive decrease of the surface tension. It is quite remarkable that in 2 minutes the drop can be of such amplitude as 2 dynes. In the three cases shown in

[3] Ferguson, A., *Science Progr. 20th Cent.*, 1914–15, ix, 428.

[4] Made by the Central Scientific Co. of Chicago.

Table I, the drop was very nearly 2.1 dynes. In 20 minutes, the surface tension was decreased by 4.1 and 3.3 dynes for the old dogs, and by 5.6 dynes for the very young dog. All three reached their equilibrium at about the same value.

If the serum is stirred at the end of the experiment, that is, when the surface tension seems to have attained a stable value, the homogeneity of the serum is momentarily reestablished, the surface tension rises

TABLE I.

Serum 1020 (Dog 12 Yrs. Old) (Text-Fig. 1).
Temperature 25°C.

Time.	0	2 m.*	6 m.	12 m.	20 m.	30 m.
Surface tension, *dynes*.....	59.6	57.5	56.9	56.1	55.5	55.5

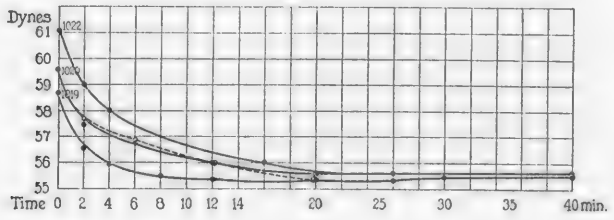
Serum 1022 (Dog 1 Yr. Old) (Text-Fig. 1).
Temperature 23°C.

Time.	0	2 m.	6 m.	16 m.	25 m.	40 m.
Surface tension, *dynes*.....	61.1	59.0	57.8	56.0	55.6	55.6

Serum 1019 (Dog 12 Yrs. Old) (Text-Fig. 1).
Temperature 23°C.

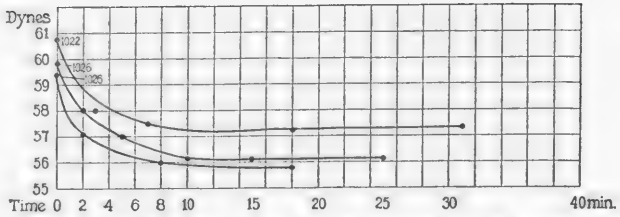
Time.	0	2 m.	4 m.	8 m.	10 m.	20 m.	25 m.	30 m.	40 m.
Surface tension, *dynes*.	58.7	56.5	56.0	55.5	55.4	55.4	55.5	55.4	55.6

* In Tables I to X, the abbreviations h., m., and s. are used for hours, minutes, and seconds.

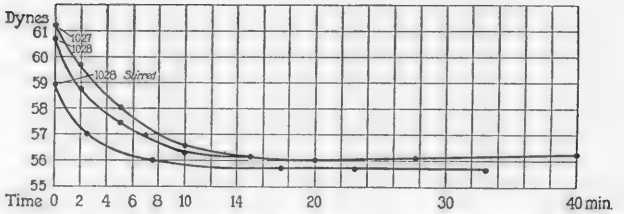


——— Observed curve.
- - - - - - - - Calculated curve.

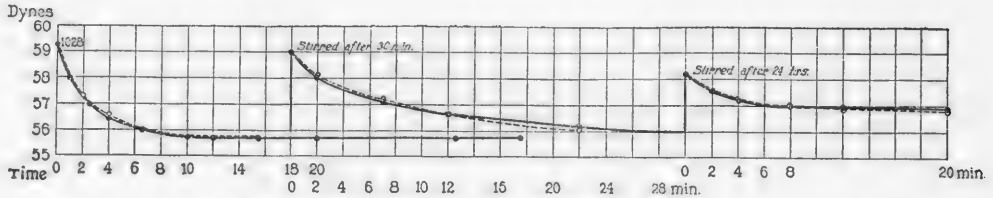
TEXT-FIG. 1. Modification of the surface tension of serum in function of the time.



TEXT-FIG. 2. Modification of the surface tension of serum in function of the time. Two samples of the same serum (No. 1026) were kept 4 days in test-tubes, the first in the ice box (initial value 59.4 dynes), the second at room temperature (22°C.; initial value 59.8 dynes).



TEXT-FIG. 3. Modification of the surface tension of serum in function of the time.



TEXT-FIG. 4. Effect of stirring on the surface tension of serum.

immediately, and the liquid recovers its property of forming another film. However, something is changed; the surface tension immediately after stirring does not reach its original value, and generally does not drop as much or as rapidly (Table II and Text-fig. 3). Text-fig. 4 shows plainly the change in the amplitude and in the rate of the phenomenon; the slope of the third curve (after 24 hours) is decidedly less marked; $\frac{d\gamma}{dt}$ decreases progressively.

TABLE II.

Serum 1027 (Dog 8 Yrs. Old).
Temperature 24°C.

Time.	0	2 m.	4 m.	8 m.	13 m.	23 m.	33 m.	4 h
Surface tension, *dynes*.....	59.8	58.5	57.9	57.1	56.5	56.5	56.3	56.3

Same serum stirred after 4 hrs., 59.0 dynes.

Serum 1028 (Dog 10 Yrs. Old) (Text-Figs. 3 and 4).
Temperature 24°C.

Time.	0	2 m.	7 m.	17 m.	27 m.	37 m.
Surface tension, *dynes*.....	59.3	57.1	56.0	55.7	55.7	55.7

Same serum stirred with a glass rod a few times, after 30 min., 59.0 dynes.

Time.	0	2 m.	4 m.	9 m.	14 m.	24 m.	50 m.	2 h.	4 h.	24 h.
Surface tension, *dynes*...	59.0	58.0	58.0	57.1	56.9	56.0	56.0	55.4	56.0	55.4

Same serum stirred again, after 24 hrs., 58.2 dynes.

Time.	0	2 m.	4 m.	8 m.	20 m.	30 m.
Surface tension, *dynes*.....	58.2	57.6	57.3	57.0	56.9	56.9

The results were comparable in experiments on chicken serum (Table III).

An interesting fact, which may be considered as a good control, is that these two sera taken from similar chickens and showing at the beginning a difference of only 0.2 dyne in their surface tension have kept this difference practically constant during 50 hours, and after

TABLE III.

Serum 1053 (Chicken).
Temperature 25°C. Oct. 11, 1921.

Time.	0	2 m.	4 m.	6 m.	11 m.	16 m.	26 m.	41 m.	56 m.
Surface tension, *dynes*.	62.0	61.2	61.0	61.0	60.7	60.0	58.0	57.0	57.0

Temperature 25°C. Oct. 12, 1921.

Time.	20 h.	Stirred.	24 h.
Surface tension, *dynes*	54.0	57.5	53.0

Temperature 25°C. Oct. 13, 1921.

Time.	44 h.	Stirred.	47 h.	50 h.
Surface tension, *dynes*	53.2	53.2	53.6	53.6

Serum 1054 (Chicken).
Temperature 25°C.

Time.	0	3 m.	6 m.	11 m.	20 m.	35 m.	1 h. 35 m.	16 h.
Surface tension, *dynes*	62.1	61.5	61.2	60.2	60.0	59.5	58.0	54.2

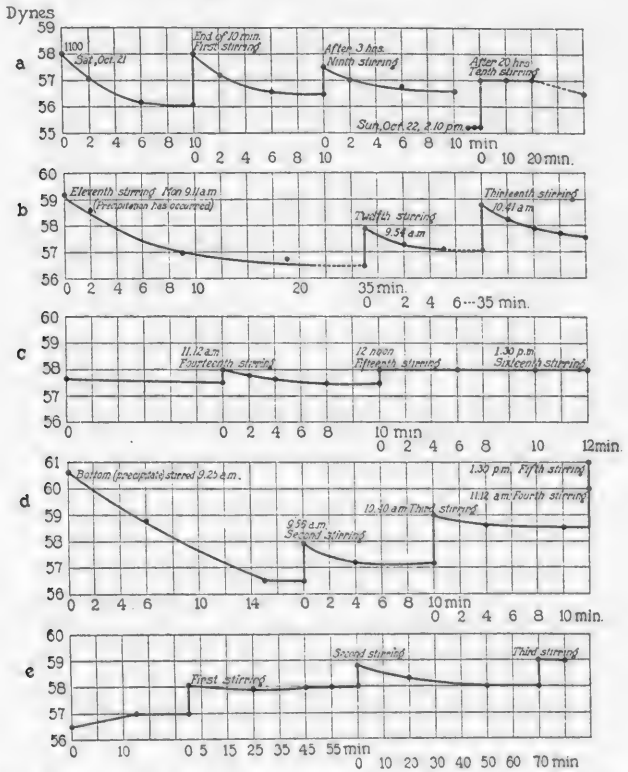
Serum stirred after 16 hrs., 58.0 dynes.

Time.	0	2 m.	12 m.	22 m.	38 m.	1 h. 10 m.	1 h. 40 m.	3 h. 55 m.	6 h.	7 h.
Surface tension, *dynes*.	58.0	58.0	57.1	56.9	56.0	55.0	54.6	53.2	54.6	54.6

Total time elapsed, 24 hrs. On Oct. 13, 1921, after 40 hrs., 53.0 dynes. Stirred, 57.0 dynes.

Time.	0	3 h.	5 h. 30 m.
Surface tension, *dynes*	57.0	54.0	53.9

being reactivated by stirring more than once. At the end of about 20 hours, the first was 54.0, and the second 54.2; at the end of 24 hours (after stirring), 53.0 and 53.2; at the end of 50 hours, 53.6 and 53.9, the difference here having become 0.3.



TEXT-FIG. 5, *a* to *e*. (*a* to *d*) Effect of stirring on the surface tension of serum. (*e*) Behavior of a control sample of the same serum, left unstirred during the 2 days, and stirred on Oct. 23, at 4 p.m., for the first time (precipitate at the bottom). Original value on Oct. 21, 56.5 dynes.

The rise in surface tension which follows the redissolution of the liquid surface layer by stirring is a general phenomenon with all clear, fresh sera. The progressive decrease in the activity, which can be compared graphically to a damping effect, seems constant also (Text-fig. 5, *a* to *e*). But it often happens that after 24 hours the property

TABLE IV.

Serum 111 (Rabbit).
Temperature 23°C.

Time.	0	2 m.	15 m.	25 m.	55 m.	1 h. 35 m.
Surface tension, *dynes*	57.5	56.5	55.5	55.4	54.7	54.6

Serum stirred after 1 hr. and 35 min., 56.9 dynes.

Time.	0	2 m.	6 m.	11 m.	26 m.	44 m.
Surface tension, *dynes*	56.9	56.1	55.5	54.6	55.0	54.7

After 2 hrs. and 20 min., the surface tension was 54.7 dynes. The next morning, after 18 hrs. and 20 min., it was 53.6 dynes. Stirred, it rose to 55.1 dynes.

Time.	0	2 m.	10 m.	1 h. 15 m.	2 h. 15 m.	2 h. 20 m.
Surface tension, *dynes*	55.1	55.0	55.0	55.1	55.0	55.0

Serum stirred at beginning of experiment, 55.1 dynes. Stirred after 2 hrs. and 20 min., 55.0 dynes.

Serum 1060 (Dog).
Temperature 23°C.

Time.	0	2 m.	6 m.	13 m.	23 m.	38 m.	2 h. 8 m.	3 h.	3 h. 15 m.
Surface tension, *dynes*.	59.3	57.5	56.8	56.0	55.5	55.4	56.0	56.0	56.0

Serum stirred after 3 hrs. and 15 min., 58.0 dynes.

Time.	0	2 m.	5 m.	10 m.	40 m.
Surface tension, *dynes*	58.0	57.1	57.0	56.7	56.0

Time.	0	2 m.	6 m.	14 m.	19 m.	50 m.	1 h. 20 m.
Surface tension, *dynes*	56.2	55.6	55.6	55.6	55.6	55.6	56.0

After 18 hrs. the surface tension was 54.2 dynes; stirred, 56.0 dynes; stirred again after 19 hrs. and 20 min., 56.0 dynes.

is completely lost. The serum is inactivated from this standpoint and its surface tension remains constant (Table IV). As will be observed, the decrease still continued after the 4th hour, and over night it dropped from 56 to 54.2 dynes. At the end of the experiment, the serum became too viscous and had to be discarded.

Action of Heat.

Time is not the only factor which inhibits this activity of the serum. Heat acts in the same way; it may not act definitively by destroying

TABLE V.

Serum 1020 (Dog; Clear, Viscous).
Temperature 25°C. Heated for 20 Hrs. at 55°C.

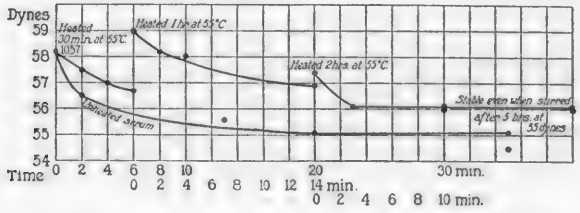
Time.	0	2 m.	8 m.	18 m.	33 m.
Surface tension, *dynes*	56.0	56.0	56.0	55.7	55.8

Serum 1021 (Dog; Cloudy).
Temperature 24°C. Heated for 20 Hrs. at 55°C.

Time.	0	2 m.	5 m.	11 m.	20 m.
Surface tension, *dynes*	57.5	57.1	57.3	57.3	57.3

Serum 1022, Control (Dog; Clear).
Temperature 24°C. Unheated.

Time.	0	3 m.	6 m.	11 m.	17 m.	31 m.
Surface tension, *dynes*	60.8	58.0	57.5	57.4	57.1	57.3



TEXT-FIG. 6. Action of heat on the surface tension of serum.

the substance, but the behavior of the serum is modified profoundly at the start, as will be seen in Table V.

TABLE VI.

Serum 1057 (Dog; Clear) (Text-Fig. 6).
Temperature 24°C. Heated for 30 Min. at 55°C.

Time.	0	2 m.	4 m.	8 m.
Surface tension, *dynes*	58.2	57.5	57.0	56.7

Temperature 24°C. Heated for 1 Hr. at 55°C.

Time.	0	2 m.	4 m.	14 m.
Surface tension, *dynes*	59.0	58.2	58.0	56.9

Temperature 24°C. Heated for 2 Hrs. at 55°C.

Time.	0	3 m.	5 m.	10 m.	25 m.	55 m.
Surface tension, *dynes*	57.4	56.1	56.1	56.1	56.1	56.1

Serum stirred after 55 min.; surface tension 61.0 dynes.

The Same Sample, after 20 Hrs. Temperature 24°C.

Time.	0	2 m.	9 m.	19 m.	34 m.
Surface tension, *dynes*	61.0	60.7	60.0	59.5	59.2

Temperature 24°C. Heated for 3 Hrs. at 55°C.

Time.	0	10 m.	45 m.	1 h. 15 m.	2 h. 15 m.	3 h.
Surface tension, *dynes*	59.3	57.0	57.0	57.4	57.5	57.4

Apparently, at least 5 hours heating at 55°C. were necessary to inactivate the sera completely, although after 2 hours the activity was reduced, as shown by Table VI and Text-fig. 6. In this case, the control itself was inactivated after 24 hours without heating.

Effect of Precipitation of Insoluble Substances in the Serum.

All the sera in which a precipitation of insoluble substances had occurred, in the form of a coarse colloidal suspension showing a tendency to settle out spontaneously after a few hours standing, were devoid of any activity (Table VII). But if the precipitate was allowed to settle, after the liquid had been stirred, the surface tension was decreased.

TABLE VII.

Serum 1059 (Dog; Cloudy; Precipitate in Suspension).
Temperature 23°C.

Time.	0	2 m.	5 m.	32 m.
Surface tension, *dynes*	53.8	53.8	53.6	53.6

When a serum is allowed to dry entirely and is redissolved in plain water, it still manifests the same activity, although to a smaller extent (Table VIII and Text-fig. 7).

TABLE VIII.

Serum 1027 (Dog). Dried and Dissolved in Water (Text-Fig. 7).
Temperature 24°C.

Time.	0	2 m.	5 m.	12 m.	27 m.	42 m.	1 h. 27 m.	2 h. 50 m.
Surface tension, *dynes*	58.0	57.2	57.2	56.9	56.1	56.2	56.2	56.1

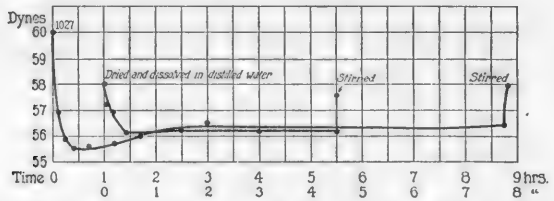
Serum 1027, Control.
Kept at Room Temperature for 48 Hrs. (23°C.).

Time.	0	2 m.	4 m.	7 m.	12 m.	22 m.	45 m.	1 h. 15 m.	1 h. 30 m.	2 h.	2 h. 15 m.	2 h. 30 m.	2 h. 45 m.	3 h. 45 m.	8 h.
Surface tension, *dynes*	60.0	57.5	57.0	56.1	55.9	55.5	55.6	55.7	56.0	56.1	56.2	56.5	56.2	56.5	56.5

Same serum stirred, 58.0 dynes.

Great care is necessary when taking the measurements, for the slightest agitation of the surface causes the film to dissolve, with a

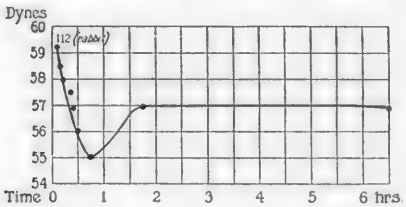
consequent rise in the surface tension. The first series of measurements was more carelessly done, and gave results which did not check with the later ones. We had to make a set of very careful measurements in which the liquid was absolutely undisturbed, to



TEXT-FIG. 7. Surface tension of fresh serum, and of the same sample dried and dissolved in water.

find out the reason for such unaccountable facts as a spontaneous rise after a drop, such as that shown in Text-fig. 8.

Text-fig. 5, *e* illustrates the damping effect again, and also shows that the progressive inactivation is mainly function of the time and



TEXT-FIG. 8. Spontaneous rise in surface tension of serum after a fall. The rise after 40 minutes and after 6½ hours was due to the careless handling of the watch-glass containing the serum.

is but little affected by stirring. It illustrates also the progressive increase—reverse phenomenon—which takes place after each stirring, when precipitation has occurred in the serum. This is probably due, as pointed out elsewhere, to the formation of a membrane or semisolid film in the surface layer (see also Text-fig. 5, *d*).

II.

DISCUSSION.

The hypothesis of surface coagulation, or the formation of an homogeneous solid film, does not seem to us to account for any of the phenomena studied. First, there is no sign, optically, of such a film. Moreover, a solid film would increase, rather than decrease, the surface tension; and finally, it would not account for the damping of the phenomenon, in function of the time. Besides, by very light stirring the tension rises again, and most of the sera experimented upon remained perfectly clear, although their viscosity increased, even after 48 hours.

An objection may be made that temperature and evaporation (concentration) play a part in this phenomenon. But a decrease in temperature, which actually occurs as soon as a liquid is spread over a larger surface, would mean an increase in surface tension, whereas we observe the opposite. Concentration following evaporation requires a good deal of time; after 20 minutes, samples were tested by measurements of their cryoscopic point and did not show any marked concentration. As the phenomenon usually takes place in less than 15 minutes, it may be concluded that it can only be affected by the change in the concentration of the surface layer. With highly concentrated sera, evaporated *in vacuo* and reduced to one-third of their original volume, the figures were practically the same.

A few hypotheses are hence possible. Either the drop in surface tension is due to a chemical change and adsorption of the resulting substances in the surface layer, or to an adsorption of bodies generated or contained in the bulk, or else to modifications in the arrangement of the group molecules in the surface layer, or possibly to a combination of these causes. Without making any attempt to discuss the structure of the surface layer, the facts may be accounted for in the case of the first hypothesis by assuming that the huge, fragile protein molecules begin to disintegrate and yield substances acting on surface tension as soon as they are no longer entirely surrounded by others in the liquid, on account of the change in potential energy. In the process of breaking down, they may also liberate progressively

other molecules, the accumulation of which would inhibit the phenomenon by concentrating in the surface layer and decreasing definitively the surface tension.

But it is not necessary to assume that the substances which lower the surface tension are produced in the surface layer. Indeed, the reaction occurs as if these substances came slowly from the bulk of the liquid. An exceedingly slight change in the concentration of certain substances in the serum could explain the facts. But there must be some chemical change to account for the damping effect, and for the inactivation, unless it is attributed to adsorption by colloidal particles. This being plainly a case of adsorption, let us consider what the differences are between this phenomenon and the ordinary phenomenon of adsorption.

In the ordinary case of adsorption, when a substance lowering the surface tension is added to the liquid the value for the surface tension corresponds to the concentration. Equilibrium is usually rapidly established with respect to the quantity adsorbed in the surface layer in excess of the quantity in the bulk, and the surface tension may reach a very low value.

In the present case, on the contrary, all constituents are coexistent, so that, at first sight, the concentration seems constant, as the substance lowering the surface tension exists normally in the serum. But either a reaction or a migration takes place in function of the time, which progressively changes the ratio of concentrations between the bulk and the surface layer. From 10 to 20 minutes are required to reach the equilibrium, which takes place generally between the limits 53 and 56 dynes, never below 53.

In the ordinary case of adsorption, the time taken for attainment of equilibrium is shortened by shaking. In the case of serum, conversely, shaking stops the process, destroys the film, and brings back the original surface tension, almost to its former value. The time required for attainment of equilibrium is not shortened, but lengthened.

Why substances lowering surface tension remain adsorbed in the surface layer, instead of remaining in the bulk as do the bodies which raise surface tension, is theoretically accounted for by the dynamical

theory of solutions, as developed mainly by Gibbs[5] and Thomson.[6] It may be of interest to the reader to recall this theory briefly.

The most stable arrangement of any solution must be one accompanied by minimal surface tension. This follows from the simple study of Gibbs' equation:

$$c\frac{d\gamma}{dc} = -u\frac{dp}{dc} \tag{1}$$

in which u is the quantity adsorbed, p the osmotic pressure, c the concentration, and γ the surface tension. For dilute solutions, the osmotic pressure is given by the equation

$$p = RT \tag{2}$$

in which R is the constant of gases and T the absolute temperature. Therefore,

$$dp = RTdc \tag{3}$$

By introducing this value in (1),

$$u = -\frac{c}{RT}\cdot\frac{d\gamma}{dc} \tag{4}$$

As stated above, u is precisely the adsorption; that is, the difference between the concentrations of the surface layer and the bulk. It is called positive when the concentration in the surface layer is greater, and negative when it is smaller than in the bulk. $\frac{d\gamma}{dc}$ is the differential coefficient of the function connecting surface tension and concentration, and is positive if γ and c increase together and negative if γ decreases with increasing c. Therefore, the whole product on the right hand of the equation will be *negative* in the first case (when the solute *increases* surface tension) and *positive* in the second (when the solute *reduces* surface tension). This means a lower concentration in the surface layer (smaller amount adsorbed, u) in the former case, and a higher concentration in the surface layer in the latter; R, T,

[5] Gibbs, W., *Tr. Conn. Acad.*, 1878, iii, 380; Scientific papers, London, 1906.

[6] Thomson, J. J., Applications of dynamics to physics and chemistry, London, 1888, 191.

and c being necessarily positive, the sign of u depends only on that of $\frac{d\gamma}{dc}$.

This explains why Gibbs' law is often given under the following form. A small amount of dissolved substance may reduce the surface tension considerably, but can only increase it slightly.

It is possible to account not only for the decrease in surface tension represented by the first curves, but also for the damping of the phenomenon, by assuming that some kind of chemical or physicochemical change is undergone by the substances in solution in the serum, in function of the time. The action of these substances, which would accumulate slowly, would be superimposed on that of the bodies acting normally, and would progressively lower the initial value of the surface tension. The presence of these new substances, whatever they are, would forcibly change the physicochemical qualities of the serum. Hence the curve representing this second stage of the phenomenon is slightly modified. The rate of the lowering of surface tension is slower, owing probably to the increased viscosity of the serum with time. When ten stirrings are made within 100 minutes, followed by a lowering of surface tension, the change in the curves, that is, in the rate of decrease, is not very different from that which would have been obtained had the serum been stirred only once at the end of the 100 minutes. Time acts more efficiently than stirring.

When a serum becomes old, precipitation occurs in the surface layer where the concentration is higher, and insoluble substances are made out of soluble ones. These ultramicroscopic particles agglomerate little by little into larger ones, until they are so heavy that they precipitate. They have a tendency to transform the surface layer into a semisolid, semiliquid layer with higher surface tension (Experiment 1059, Table VII). In order to prevent confusion we shall refer hereafter to the reaction which takes place for the first time, as soon as the serum is exposed to the air, as the first reaction. After the first stirring, it will be the second reaction, and so on. There is no doubt that one reaction takes place as soon as the blood is removed from the body, and another every time the serum is poured from one tube to another. But in such cases, the surface exposed is small and when not moved is protected by the first film formed. Besides, we

must arbitrarily choose a starting point, and mathematical study of the phenomenon shows that our assumption is probably correct.

III.

Mathematical Expression of the Phenomenon.

An attempt was made to establish a general equation on a purely empirical basis, which would express the phenomenon in all cases, before and after stirring, with only one coefficient. We found that the exponential formula

$$\gamma = \gamma_o e^{-Kt^{\frac{1}{3}}} \tag{5}$$

expressed the facts very closely (in the case of dog and rabbit serum), until the phenomenon became asymptotic to a parallel to the axis of time, the ordinate of which is generally around 55.5 dynes. After 10 to 20 minutes, the curve becomes parallel to the axis of time and the phenomenon stops. This fact may be expressed, if required, simply by the addition of a linear term to formula (5). We have, then,

$$\gamma = \gamma_o e^{-Kt^{\frac{1}{3}}} + At - B \tag{6}$$

t being the time, γ the surface tension at the time t, γ_o the surface tension at the beginning of the experiment, K, A, and B three constants. In fact, the constants A and B do not modify the law, and the corrective term is only required for the first reaction. After stirring, the first simple formula (5) suffices to account for the facts with close agreement practically to the point when equilibrium is attained. Indeed, the phenomenon is characterized by only one constant, K, generally equal to 0.01, for average old dogs; B is equal to 1.6 (in the example chosen—Serum 1020, Table X), and A to 0.1.

There may be other equations by means of which this law can be expressed, as always occur in cases of logarithmic curves. But the advantage of this exponential form is that it requires only one constant, the meaning of which is clearer, and that it is short and, as is well known, very easy to calculate under the form

$$\text{Log } \gamma = \text{Log } \gamma_o - K\sqrt{t} \tag{7}$$

Tables IX and X show the agreement between observed and calculated figures. The two curves in Table X were calculated with the simple formula (5). It will be noticed that the constant K decreased rapidly after each stirring, indicating that the serum becomes saturated, or inactivated, after a certain time.

TABLE IX.

Serum 1028 (Dog).
First Reaction.
$K = 0.01$

Time.	0	2 m.	4 m.	6 m. 5 s.	16 m.	20 m.	40 m.
Surface tension observed, *dynes*.....	59.3	57.2	56.5	56.0	55.7	55.7	55.7
" " calculated, *dynes*....		57.3	56.6	56.0	55.7	55.7	55.7

These figures are obtained from the 16th min. on, with the complete formula (6). $C = 0.17$ and $A = 1$.

Second Reaction; Stirred after 30 Min. (Text-Fig. 4).
$K = 0.005$

Time.	0	2 m.	7 m.	12 m.	22 m.
Surface tension observed, *dynes*.....	59.0	58.0	57.1	56.7	56.1
" " calculated, *dynes*.....		58.1	57.2	56.7	56.0

Third Reaction; Stirred after 24 Hrs. (Text-Fig. 4).
$K = 0.00325$

Time.	0	2 m.	4 m.	8 m.	12 m.
Surface tension observed, *dynes*.....	58.2	57.6	57.3	57.0	56.9
" " calculated, *dynes*.....		57.6	57.3	57.0	56.8

The progressive decrease in surface tension after each stirring is shown again in Text-fig. 5, *a*. As stated before, this phenomenon seems to happen only when there is no precipitation in the liquid; as soon as a certain amount of precipitation has taken place, the surface tension, after stirring, rises higher than its former values.

It may seem very premature to try to apply any theoretical formula to this phenomenon, considering the enormous complexity of the

liquid studied, of whose constitution we are so ignorant. However, an interesting fact leads us to believe that it may some day be done successfully. As already noted this phenomenon is one of adsorption in the surface layer. Mere reasoning makes this clear. But a comparison of one of our curves with a curve obtained by Lewis[7] for the adsorption of sodium glycocholate by a surface of paraffin oil shows a

TABLE X.

Serum 1020 (Dog under 1 Yr. of Age).
K = 0.0065

Time.	0	2 m.	6 m.	12 m.	20 m.	25 m.
Surface tension observed, *dynes*.....	59.6	57.5	56.9	56.1	55.5	55.5
" " calculated, *dynes*.....		57.7	56.9	56.0	55.3	55.3

In this case, the coefficient is much smaller.

Serum 1022 (Old Dog).
K = 0.01

Time.	0	2 m.	6 m.	16 m.	25 m.
Surface tension observed, *dynes*.....	61.1	59.0	57.8	56.0	55.6
" " calculated, *dynes*.....		59.0	57.75	55.8	

remarkable identity despite the entirely different conditions (Text-figs. 9 and 10). A few formulas have been given for expressing the phenomenon of adsorption in function of the quantity adsorbed and of the concentration. It is clear that they could not be used in our case. Among these we may mention the formula quoted by Duclaux[8] and that of Freundlich,[9] inaccurate at higher concentrations as shown by Schmidt,[10] who gives a rather complicated exponential formula[11]

[7] Lewis, W. C. McC., *Proc. Physic. Soc.*, 1909, xxi, 150; *Phil. Mag.*, 1909, xvii, 466; *Z. Chem. u. Indust. Kolloide*, 1909, v, 91; also cited by Willows, R. S., and Hatschek, E., Surface tension and surface energy, and their influence on chemical phenomena, Philadelphia, 1915, 41.

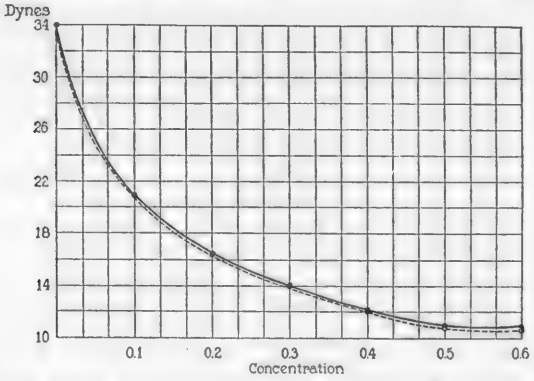
[8] Duclaux, J., Les colloïdes, Paris, 1920, 148.

[9] Freundlich, H., Kapillarchemie: Eine Darstellung der Chemie der Kolloide und verwandter Gebiete, Leipsic, 1909, 146.

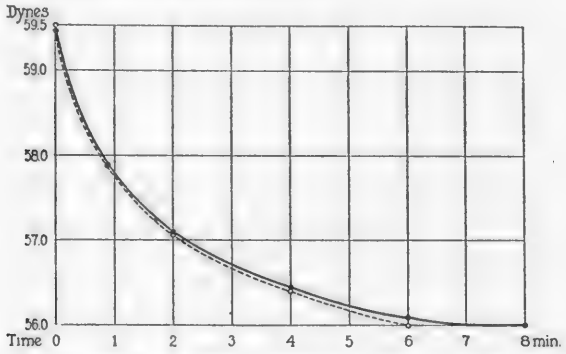
[10] Schmidt, G. C., *Z. physik. Chem.*, 1910, lxxiv, 699.

[11] Schmidt, G. C., *Z. physik. Chem.*, 1911, lxxvii, 641.

with two constants. This equation applies well to the adsorption of liquids by solids, and Arrhenius[12] has pointed out that it could be



TEXT-FIG. 9. Action on surface tension of adsorption of sodium glycocholate by paraffin oil (Lewis; adsorption isotherm).



TEXT-FIG. 10. Action on surface tension of adsorption in the surface layer of serum.

simplified. A formula in function of surface tension was given by Sentis[13] in 1897, and another by Valson in 1872.[14] The last two only

[12] Arrhenius, S. A., Conférences sur quelques thèmes choisis de la chimie physique, Paris, 1912, 31.

[13] Sentis, Recueil de constantes physiques, Paris, 1913, 122.

[14] Valson, cited by Morgan, J. L. R., and McKirahan, W. W., *J. Am. Chem. Soc.*, 1913, xxxv, 1759.

apply to inorganic salts, which raise the surface tension and therefore are not adsorbed in the surface layer. Of course, the curve of Lewis is not drawn in function of the time, but in function of the concentration of the solution. In our case, the concentration is constant, but we can admit that the relative concentration is proportional to the time, since the solute which lowers the surface tension is either formed in the free surface layer as soon as it exists, or travels through the bulk to it, in function of the time.

The perfect identity of the curves is emphasized by the fact that the new equation (5), which we proposed for the phenomenon studied in this paper, applies remarkably well to that of Lewis (Table XI). This gives a greater character of generality to this formula, which

TABLE XI.

Agreement between Figures Observed and Calculated for the Lowering of Surface Tension at the Interface between a Solution of Sodium Glycocholate and Paraffin Oil, in Function of the Concentration (Text-Fig. 9).

$K = 0.222$

Concentration, *per cent.*	0	0.1	0.2	0.3	0.4	0.5	0.6
Surface tension observed (Lewis), *dynes*	34.0	21.0	16.5	14.0	12.2	11.0	10.9
Surface tension calculated ($\gamma = \gamma_0 e^{-Kc^{\frac{1}{3}}}$), *dynes*		20.2	16.5	14.0	12.2	10.9	9.7

may be applied to the phenomenon studied by Traube,[15] Lewis,[7] Donnan and Barker,[16] Langmuir,[17] and others, simply by changing t to c; namely,

$$\gamma = \gamma_0 e^{-Kc^{\frac{1}{3}}} \tag{8}$$

Toward the end of the curve, it becomes parallel to the abscissa of ordinate 10, as a state of equilibrium is attained, and, for the same reasons as expressed above, the calculated data deviate from those observed (Text-fig. 9).

[15] Traube, J., *Kolloidchem. Beihefte*, 1911–12, iii, 237.

[16] Donnan, F. G., and Barker, J. T., *Proc. Roy. Soc. London, Series A*, 1911, lxxxv, 557.

[17] Langmuir, I., *J. Am. Chem. Soc.*, 1917, xxxix, 1848.

Besides, as the measurements of Lewis were made with the drop weight method, it shows that the method used in our experiments on colloidal substances is as reliable as the most accurate and time-consuming methods used by Lewis.

To give an idea of the order of magnitude of the excess of concentration in the surface layer, since we cannot measure u directly, we may use Gibbs' formula tentatively, without laying emphasis upon the figures given here. Assuming the total concentration in the bulk of a serum to be 9.9 per cent after 1 minute (electrolytes and non-electrolytes), and referring to the formula

$$u = -\frac{c}{RT} \cdot \frac{d\gamma}{dc} \tag{4}$$

we have the values

$$c = 9.9;\ R = 83.2 \times 10^6;\ T = 293°;\ \frac{d\gamma}{dc} = \frac{d\gamma}{dt} = 2$$

$\frac{d\gamma}{dc}$ = the tangent of the angle of the tangent to the point of abscissa 1 with the axis of the abscissæ.

$$u = 0.81 \times 10^{-7} \text{ gm. per sq. cm.}$$

The free surface being about 10 sq. cm., it gives a total amount, roughly, of $\frac{1}{1{,}000{,}000}$ gm. of substance adsorbed in the surface layer; but as we deal with colloidal solutions, this figure is almost certainly wrong.

As a point of comparison, it may be said that for sodium glycocholate Lewis found experimentally that $u = 3.6 \times 10^{-6}$ instead of about 1.0×10^{-7}, the theoretical value; and Donnan and Barker, using nonylic acid as solute, obtained for u, 0.95×10^{-7}, instead of 0.55×10^{-7}, calculated.

We must not forget that the theoretical values of u (quantity adsorbed) in certain cases were twenty to thirty times smaller than the experimental values. This may be due partly to great difficulties in the technique. The discrepancy is still more marked for colloidal substances. Langmuir[17] has insisted upon the differences existing between theoretical and experimental data.

Further experiments are now being carried out on this subject.

IV.

CONCLUSIONS.

1. Over 3,000 measurements of surface tension of sera have been made with the ring method, and they have yielded a new phenomenon, the spontaneous and rapid decrease of the surface tension of a serum in function of the time.

2. Generally, after 10 minutes the surface tension reaches a value which is practically constant. At least, the decrease is very much slower. After stirring, a rise occurs and a similar phenomenon takes place; but stability is not obtained as rapidly, requiring about 25 minutes. By stirring again, the same thing happens repeatedly, the slope of the curve being less marked each time, the rise in surface tension being slightly below each previous value, and the phenomenon undergoing a sort of damping.

3. An equation was established which expresses the experimental facts with an accuracy of about 0.2 per cent. It applies to the whole phenomenon, before and after stirring. It has only one characteristic constant,

$$\gamma = \gamma_0 e^{-Kt^{\frac{1}{3}}}$$

This formula, by simply changing t to c (concentration), expresses satisfactorily in general the phenomenon of adsorption in the surface layer; that is, the decrease in surface tension in function of the concentration.

4. Prolonged heat, at 55°C., and time seem to inhibit this phenomenon.

5. When precipitation occurs in a serum, the bottom of the liquid, which contains the precipitate, has the highest surface tension. When stirred, the surface tension rises a little every time. The upper part, clear, with lower surface tension, shows the reverse phenomenon; after every stirring, the surface tension becomes a little lower.